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JUN 23 1950

CANCER RESEARCH

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THE OFFICIAL ORGAN OF THE

AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.

Published by THE UNIVERSITY OF CHICAGO PRESS

CANCER RESEARCH

This journal is sponsored by The American Association for Cancer Research, Inc.; The Anna Fuller Fund; Cancer Research Division, Donner Foundation, Inc.; The Jane Coffin Childs Memorial Fund for Medical Research; The Elsa U. Pardee Foundation; and The American Cancer Society.

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Business communications, remittances (in United States currency or its equivalent), and subscriptions should be addressed to THE UNIVERSITY OF CHICAGO PRESS, 5750 Ellis Avenue, Chicago 37, Illinois. All other communications should be addressed to Harold P. Rusch, M.D., McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wisconsin.

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Entered as second-class matter, February 15, 1949, at the post office at Chicago, Ill., under the Act of March 3, 1879.

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CANCER RESEARCH

VOLUME 10 JUNE 1950 NUMBER 6

Comments on Recent Experiments with Frozen and Dried Tissue as Evidence for the Virus Etiology of Tumors

ERICH HIRSCHBERG, M.S.,* AND HAROLD P. RUSCH, M.D.

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wisconsin)

One of the tasks confronting investigators in any field is an occasional re-evaluation of the experimental work on which the basic theories in that field depend. A special impetus is given to such an endeavor when new data are collected and cited as conclusive evidence for or against any of these theories. The concept that the etiology of tumors involves the presence of viruses is one of the oldest theories on cancer: its popularity has varied from time to time and from investigator to investigator. With the unequivocal demonstration of the viral nature of the causative agents of a number of tumors, the main question has become whether *all* tumors are virus-induced. Ultrafiltration experiments have given an essentially negative answer, since it has not been possible to transplant most mammalian tumors by means of cell-free filtrates (16, 17).

The question, however, has been vigorously reopened by the recent work of Gye and his associates in London. These investigators have made extensive use of freezing and drying technics and have indicated that the successful transplantation of mammalian tumor tissue after these treatments, which are considered to have killed all the cells, is convincing evidence for the viral nature of the transmitting agent. These findings and conclusions have created widespread interest.

The present review brings together some of the available facts bearing on this problem. No position is taken on the merits of the virus theory in general. The central point of this review is to discuss the assumption of Gye and his collaborators that freezing and drying kill all the cells of a tissue and to inquire whether it can be safely concluded at this point that these technics provide unequivocal proof for the concept that the transplantation of mammalian tumors involves viruses and not intact cells. It is also hoped that this review may stimulate further investigations on this problem by indicating some of the approaches which may not yet have gained sufficient recognition.

Summary of the findings of Gye et al.—This work has been presented in a number of papers by Craigie, Gye, Mann, and others (9, 10, 14, 15, 22–25) and commented upon by Andrewes (1) and in two editorials in the British Medical Journal (2, 3). The conclusions of Gye and his associates may be briefly summarized as follows:

Normal embryonic tissue could not be successfully transplanted, and no normal cells could be found by histological examination after an hour's exposure to −79° C. Normal embryonic tissue was also killed when treated with glycerol and stored at 40° C., while fine emulsions of frozen neoplastic tissue made with distilled water, 40 per cent glycerol or 40 per cent glucose caused cancer. A sarcoma suspension frozen in dextrose and 40 per cent glycerol was highly active after 253 days. Three mouse sarcomas, one induced chemically and the others spontaneous, were successfully propagated with tissues dried completely after freezing; some mouse sarcomas even retained their activity after drying without preliminary freezing. The continuing cause of these tumors was postulated to be a

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Received for publication, April 4, 1950.

virus which was stable at −79° C. Mann showed that mammary carcinomas whose etiology was associated with the Bittner virus could be transplanted after freezing to −79° C. Under the usual conditions, when the virus is passed through the milk, an average of 9 months elapses before tumors appear; since tumors grew within a few weeks after freezing, she postulated that this treatment liberated an active form of the virus. The activity increased with the length of refrigeration, at least for the first 48 hours, and the cells which had been frozen induced tumors only in mammary tissue, in contrast to living tumor cells which grow in a variety of tissues. Repeated freezing and thawing of mouse mammary carcinoma tissue reduced its infective activity. Active Bittner virus obtained from this tissue by a single freezing could be dried *in vacuo* if no thawing occurred before the material was dry; such a preparation produced tumors in from 4 to 10 weeks in both male and female mice when injected into breast tissue.

The basic point conveyed by these papers is that tumor tissue, when transplanted following freezing and drying, is still capable of causing vigorous neoplastic growth in the new host, while normal cells cannot withstand these treatments. Gye *et al.* considered these facts to be proof of the viral nature of the causative agent of these transplantable mammalian tumors.

Survival of normal cells after freezing.—Perhaps the weakest link in the chain of evidence built up by Gye *et al.* is their readiness to assume that the lack of growth of normal embryonic tissue of two strains of mice after freezing is sufficient evidence for the death of all normal cells at extremely low temperatures; evidence to the contrary with other normal tissues, and under conditions different from those employed by Mann (22), is available. The following paragraphs do not purport to give an exhaustive survey of the pertinent literature but rather a summary of certain representative findings.

Breedis and Furth (7) showed that tracheal epithelium of chickens survived slow freezing to −70° C. and storage at that temperature for 327 days. Many cilia were actively motile after this treatment, but the suitability of this criterion for life was later questioned by Breedis himself (6). According to Mider and Morton (26), normal adult rat skin could be frozen rapidly to −74° C. and maintained at that temperature for 24 hours without losing its capacity to grow in the host, as determined by histologic examination. Thoennes (28) showed that muscle fibers from the sartorius of the frog, immersed in liquid air at −195° C. for periods up to 1 hour and rewarmed rapidly by immersion in Ringer's solution at 35° C., responded to electrical stimulation in 91 per cent of the cases. Klinke (20) obtained evidence by vital staining for the survival of renal, testicular, and ovarian tissues of several animal species after storage at −196° C. for several weeks. Tissue culture experiments by Klinke also showed that even short exposure to liquid nitrogen at −253° C. occasionally permitted the survival of fragments of chick embryo liver, brain, and heart. Briggs and Jund (8) subjected the ventral or dorsal skin of three inbred strains of mice to slow freezing to −78.5° C.; even when maintained at this temperature for from 1 to 24 hours, autoplastic grafts to dorsum or venter were successful in from 50 to 100 per cent of the cases. Quite recently, Blumenthal and his collaborators (4, 5) exposed the thyroid gland of guinea pigs to low temperatures and then autotransplanted it into a subcutaneous pocket in the anterior abdominal wall; 1 of 12 transplantation attempts was successful after exposure to −70° C. for 24 hours, while 8 viable thyroid grafts were obtained in 12 attempts after immersion at −190° C. for 10 minutes.

Not all attempts along these lines were successful. For example, Koose and Lemmel (21) reported that mouse skin exposed to liquid air for a short time appeared normal for 10–14 days after grafting but then always died. However, the majority of experiments in which normal tissue did not survive freezing involved embryonic tissue; the findings of Gaylord (13) with young mouse embryos, of Simonin (27) with rat, mouse, and ox embryos, and of Ferguson (12) with the smooth muscle of chick embryos may be cited. In view of the apparently greater sensitivity of embryonic tissue to freezing, it would seem particularly unfortunate that Mann (22) chose this type of normal tissue as the only control for the freezing procedures used in her recent investigations.

Growth of tumor tissue in tissue culture after freezing.—Tissue culture provides an especially useful tool for investigating the effect of low temperature on the survival of intact neoplastic cells. The successful culture of such cells following freezing is conclusive evidence that the cells were not destroyed by this treatment. The viability of cells following freezing has been determined in several experiments with variable results. Koose and Lemmel (21) were unable to find any evidence of growth attributable to intact cells when mouse carcinoma tissue was frozen in liquid air for 5 minutes. Cramer (11) was unable to cultivate normal, carcinoma, or sarcoma cells in tissue culture after repeated freezing and thawing.

On the other hand, positive results have been

obtained in the extensive studies of Klinke (18–20). Definite evidence for the presence of living cells in Ehrlich mouse sarcoma and Jensen sarcoma tissue after freezing in liquid nitrogen for 48 hours was obtained in this way. While not all attempts to culture such cells were successful, it would seem that the growth in tissue culture of even a few explants subsequent to freezing clearly indicates the survival of intact cells. Continued work along these lines is indicated.

Discussion.—A comparison of the findings of the various investigators is severely hampered by the diversity in experimental materials, technics of freezing and thawing, and criteria used to determine the presence or absence of intact cells. Nevertheless, every example of a successful survival of normal tissue to freezing and every demonstration of growth in culture of an explant of frozen tumor tissue weakens the arguments which have been advanced for the viral nature of the transmitting agent in this type of experiment.

Up to this point, the weaknesses of the position of the British investigators have been emphasized. It is also essential to discuss the more persuasive aspects of their work. These will be mentioned briefly, since many of the experiments which would permit an evaluation of these aspects from all points of view still remain to be done.

Gye and his associates rightly stress the fact that they have greatly strengthened their body of evidence by the subsequent drying of frozen cells. The experiments showing that tumor tissue suspensions can withstand treatment with 40 per cent glycerol without loss of activity also argue against the presence of intact cells in the material used for transplantation. Freezing-drying is of course widely used to preserve bacterial cultures, but no adequate information is available concerning the effect of such a treatment on normal mammalian cells of various types. Until such information becomes available, the drying of frozen tumor tissue appears to be the most promising technic to be used in these studies.

Answers to the following questions, then, should do much to establish the validity of the conclusions of Gye *et al.:* Is it possible to freeze-dry normal tissue with the technics described by the London group without abolishing their capacity to be successfully grafted into new hosts? Can tumor or normal tissue be subjected to the freezing-drying technic and subsequently grow in tissue culture? Can frozen tumor suspensions be passed, after thawing, through Berkefeld or similar filters to yield active but unequivocally cell-free filtrates? What is the effect of 40 per cent glycerol under the conditions of the British investigators on a wide variety of normal cells? Until some of these points have been elucidated, there is little hope of coming to a final decision on the correct interpretation of these findings. With the information now at hand, it would appear that this type of study has not yet proved or disproved the theory that all tumors are virus-induced.

Summary.—The data on the propagation of mammalian tumors in the absence of intact cells obtained by Gye *et al.* with freezing and drying technics are summarized and discussed. It is pointed out that a fair amount of evidence exists for the survival of normal cells after freezing. The occasional growth of tumor tissue in tissue culture after freezing further indicates that freezing technics alone are not suitable for the solution of this problem. The introduction of drying after freezing appears to provide stronger evidence against the presence of intact cells. Some questions are raised which must be answered before the data obtained by freezing-drying can be completely evaluated.

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Further Evidence for an Alteration in the Structure of a Polarographically Reducible Substance in Carcinogenesis*

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Recent evidence shows that a qualitative chemical change occurs in a substance extractable from mouse and human epidermis when this tissue becomes carcinomatous (1, 2). The separation and some properties of the substance undergoing alteration in carcinogenesis are described in this report.

EXPERIMENTAL

The material from epidermis and other tissues was extracted with ether and alcohol mixtures, as previously described (1, 2). Usually 0.5–1.0 gm. of total lipid was used for the purification of the polarographically reducible substance. The total lipid was dissolved in anhydrous peroxide-free ethyl ether and by evaporation concentrated in a 40-ml. centrifuge tube. The phospholipids were precipitated by the addition of ice-cold acetone, alone, since this procedure proved satisfactory for the problem at hand. The phospholipids were then centrifuged at 2,000 r.p.m. at 0° C. The phospholipids were then washed 3 times with ice-cold acetone, centrifuged, and the acetone-soluble fractions were combined and brought to dryness on a steam bath. The polarographically reducible substance was present in the acetone soluble fraction. The counter-current distribution procedure developed by Craig (3, 4), using nine separatory funnels, was employed for further fractionation between the following two mixtures: (*a*) 38.0 ml. of alcohol, 38.0 ml. of acetone, and 24.0 ml. of water saturated with petroleum ether, and (*b*) petroleum ether saturated with mixture (*a*).

Usually, 200–400 mg. of the acetone-soluble material were dissolved in 20 ml. of mixture (*b*) and delivered into separatory funnel No. 0. To this was added 20 ml. of mixture (*a*), and the two phases were thoroughly mixed and allowed to separate completely. The lower phase (alcohol, acetone, and water, saturated with petroleum ether) which contained the polarographically reducible substance was then run into 20 ml. of mixture (*b*) in separatory funnel No. 1, and 20 ml. of mixture (*a*) was pipetted into separatory funnel No. 0. Then, the contents of both funnels were mixed, the phases allowed to separate, and the same procedure repeated to separatory funnel No. 9, as described by Craig (3, 4). The reducible substance from epidermis and carcinoma was distributed maximally in the acetone, alcohol, and water mixture as determined polarographically, and it was almost all concentrated in separatory funnel No. 9. No other attempts were made to purify further the substance by this technic.

Since practically all the reducible substance was distributed in the alcohol, acetone, and water layer in separatory funnel No. 0 after the first extraction, the upper layer of petroleum ether in funnel No. 0 was extracted only once in the most recent experiments. Then, the bottom layer containing the reducible substance was run through all the separatory funnels containing petroleum ether saturated with the alcohol, acetone, and water mixture. The reducible substance in the lower phase of the last separatory funnel was run into a 50-ml. Erlenmeyer flask, which was placed upon the top shelf of a warm (56° C.) vacuum oven, into which had been placed, just previously, a few pieces of solid CO_2 on the bottom shelf. The solvents were then evaporated at 56° C. under partial vacuum (hereafter denoted by drying at 56° C.). The light yellow and oily-appearing dry material so obtained represented 0.01–0.02 per cent, by weight, of the original wet weight of tissue. These properties were characteristic of all the tissues extracted and reported in this paper. The yellow substance was then dissolved in cold absolute alcohol, filtered through Munktell's OB paper into a 12- or 15-ml. centrifuge tube and then concentrated to 0.5–2 ml., depending upon the amount of total lipid and the tissue extracted. To the

* This investigation was aided by grants from the Charles F. Kettering Foundation, the American Cancer Society, and the National Cancer Institute.

Received for publication, December 19, 1950.

tubes containing the substance from epidermis and carcinoma were added 3 drops of 0.02 N HCl, 0.3 ml. of 0.2 M $CaCl_2$, and then an amount of water equal to $\frac{1}{2}$ the original volume after concentrating the material. The solutions became turbid immediately, and, after standing overnight in an ice bath at 0°, the calcium salts of the fatty acids flocculated. The fatty acids are probably hydroxy fatty acids because of their solubility in alcohol. These were spun down at 2,000 r.p.m. at 0° and the supernate filtered. The polarographically reducible substance was present in the yellow filtrate, which was dried at 56° C. With slight modifications, the procedure described above was used for mouse and human epidermis, mouse squamous-cell carcinomas, mouse liver and muscle, and transplantable tumors derived from the latter two organs.

TABLE 1

BEHAVIOR OF THE REDUCIBLE MATERIAL FROM EPIDERMIS AND CARCINOMA UNDER VARIOUS CONDITIONS

Material	Control	After calcium precipitation of fatty acids	With 0.2 N NaOH for 1 hour	With 0.2 N NaOH at 100° C. for 10 minutes
	Microamperes per aliquot			
Normal mouse epidermis	3.52	3.20	3.32	3.08
Human epidermis	2.84	2.72	2.40	2.88
Carcinoma	0.67	0.60	0.60	0.60

Some properties of the purified substance obtained from normal and hyperplastic mouse epidermis and from the squamous-cell carcinoma.—The highly purified material (hereafter denoted by the fraction representing 1 to 2 per cent by weight of the total lipid) from these tissues is light yellow and oily in appearance. It is soluble in alcohol and acetone, forms a turbid suspension in water, and is insoluble in ether, petroleum ether, benzene, and other nonpolar solvents. The substance can be dehydrated to a light tan powder in an Abderhalden Pistol, with chloroform as a refluxant. However, the material is so extremely hygroscopic that liquefaction occurs before microscopic examination for crystal structure can be carried out. The same general behavior has been noted for the material extracted from the other tissues mentioned previously.

About 10–30 mg. of the purified substance were used for sodium fusion for the qualitative detection of nitrogen by the prussian blue reaction, halides by the silver chloride test, and sulphur by the formation of lead sulphide. Another sample was digested with a mixture of nitric and perchloric acids and the presence of phosphate tested by the formation of ammonium phosphomolybdate. With these tests, the substance from epidermis and carcinoma contained phosphorus, but no nitrogen, halides, or sulphur.

Stability of the reducible substance from both epidermis and the carcinoma was suggested, since extraction with solvents under aerobic or anaerobic conditions did not affect the yield. Since the material was hygroscopic, the possibility of a carbohydrate was suggested. Therefore, the behavior of the purified substance in 0.2 N NaOH at room temperature for 1 hour and at 100° C. for 10 minutes on a steam bath was studied. After neutralization with HCl, the samples were dried at 56° C., dissolved in cold alcohol, and filtered. Polarography was carried out as previously described (1, 2), and the results are expressed as microamperes per aliquot, or $\frac{1}{4}$ of the total sample (Table 1). The value for the control samples is shown in the first column, and the data in the second column reveal that the reducible substance is not precipitated as the calcium salt along with calcium salts of fatty acids. There is some decrease in diffusion current in the substance from the epidermis with NaOH at room temperature and at 100° C., but probably this diminution is not significant, due to the small amounts of material used and the handling involved. These data, and the fact that the extent and maximum of absorption in the ultraviolet was likewise not appreciably affected under similar conditions, do not suggest the presence of carbohydrate.

Behavior of the reducible substance in the ultraviolet.—A Beckman quartz spectrophotometer, model DU, was used for the ultraviolet measurements. The measurements were made in 95 per cent alcohol or in mixtures of the latter (50 per cent by volume) and 50 per cent by volume of 0.2 M HCl-KCl buffer for a final pH of 3.8, or by 50 per cent by volume of 0.2 M phosphate buffer for a final pH of 6.9, or by 50 per cent by volume of 1.0 M NH_4Cl-NH_4OH for a final pH of 9.2. The absorption spectrum of the substance from normal and hyperplastic epidermis and the carcinoma at pH 9.2 is shown in Fig. 1. The absorption maxima at pH 3.8, 6.9, and 9.2 are given in Table 2, and they are practically the same for normal and hyperplastic epidermis and decrease in the same order of magnitude with decreasing pH. On the other hand, the absorption maximum of the substance of the carcinoma is 260 mμ and is pH independent. In 50 per cent alcohol, the maximum of the substance of normal and hyperplastic epidermis is 280 mμ, while that from the carcinoma is 260 mμ. Therefore, in addition to the difference in the polarographic behavior of the substance from epidermis and carcinoma (1, 2),

there are also two differences in absorption characteristics. These latter properties confirm the existence of a qualitative chemical change in the transformation of epidermis to squamous-cell carcinoma.

Polarography of the highly purified substance.—The half-wave potentials and the proportionality between diffusion current and concentration of the reducible substance in epidermis and carcinoma have been given (1, 2). The half-wave potentials (in volts versus saturated calomel electrode, S.C.E.) of the crude and purified material in epidermis and carcinoma are shown in Table 3. There is little difference in the half-wave potentials between the crude and purified material, except in the carcinoma at the lower pH values. The fractionated reducible substance of normal epidermis shows the double wave at pH 6.4 and 6.7, and it is, therefore, probably characteristic of the pure compound as was previously postulated (1). On the other hand, the purified substance from late hyperplastic epidermis does not show a double wave at these pH values, and this fact may indicate a slight chemical change in this substance in the pre-cancerous epidermis. As with the crude material, the fractionated substance from the carcinoma has only a single wave. In addition to mouse and human epidermis, this tissue from the rat yields a substance which is indistinguishable, polarographically, from that of the former two species in the crude state.

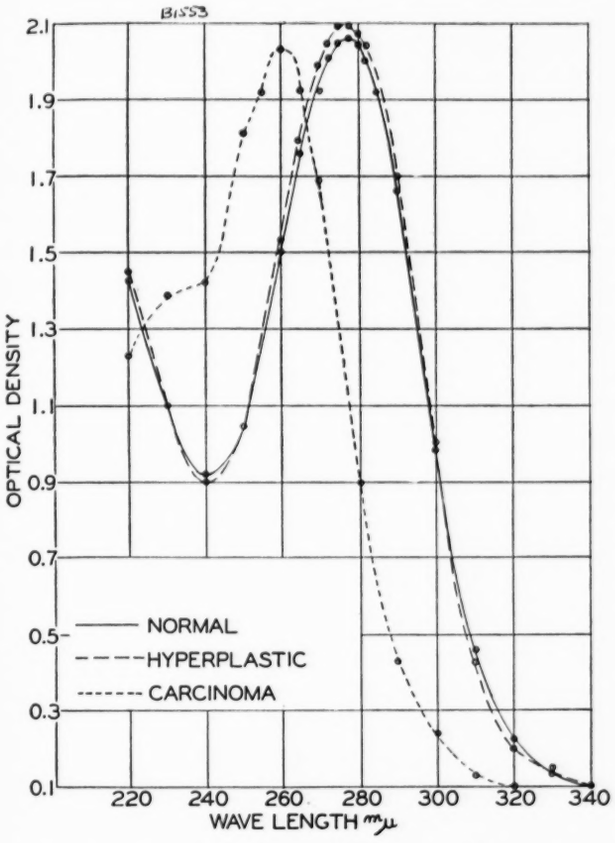

FIG. 1.—Ultraviolet absorption of the reducible substance from epidermis and carcinoma at pH 9.2 in an equal volume of 1.0 M NH_4Cl-NH_4OH buffer and an equal volume of 95 per cent alcohol.

TABLE 2

ABSORPTION MAXIMUM OF THE REDUCIBLE MATERIAL FROM EPIDERMIS AND SQUAMOUS-CELL CARCINOMA

Tissue	pH 3.8 (mμ)	pH 6.9 (mμ)	pH 9.2 (mμ)	Alcohol (mμ)
Normal epidermis	268	272	278	280
Hyperplastic epidermis	270	273	276	280
Carcinoma	260	260	260	260

Study of mouse liver and muscle and of the tumors derived from them.—The half-wave potentials in the total lipid of liver and a well differentiated transplantable hepatoma[1] and of muscle and a

[1] This hepatoma, tumor C954, arose spontaneously in mice of Dr. Cloudman's leaden strain at the Roscoe B. Jackson Memorial Laboratory. It was first transplanted in January, 1940.

TABLE 3

HALF-WAVE POTENTIALS IN VOLTS VERSUS S.C.E. OF THE REDUCIBLE MATERIAL FROM EPIDERMIS AND CARCINOMA OF MICE*

Material	Nature	Half-wave potential in volts versus S.C.E.	pH 4.3 (volts)	pH 6.4 (volts)	pH 7.2 (volts)
Normal epidermis	Total lipid	First wave	−1.40	−1.57	
		Second wave	H†	−1.65	−1.71
Normal epidermis	Purified	First wave	−1.40	−1.57	
		Second wave	H†	−1.65	−1.73
Hyperplastic epidermis	Total lipid	First wave	−1.40	−1.57	
		Second wave	H†	−1.71	−1.73
Hyperplastic epidermis	Purified	First wave	−1.40	−1.56	
		Second wave	H†	−1.71	−1.72
Carcinoma	Total lipid	First wave	−1.34	−1.47	−1.50
Carcinoma	Purified	First wave	−1.31	−1.44	−1.49

* For the dropping mercury electrode used in these experiments $m^{2/3}\ t^{1/6} = 1.924\ mg.^{2/3}\ sec.^{-1/2}$.

† Hydrogen wave.

highly anaplastic transplantable rhabdomyosarcoma[2] are practically the same (Table 4). The substance in a high state of purity from the rhabdomyosarcoma has an absorption maximum at 260 mμ which is pH independent from pH 3.8 to 9.2, but the substance of normal muscle does not absorb at 260 mμ at the same pH values. The substance of normal muscle has a broad band between 230 and 240 mμ. Although the amount of purified substance from the muscle tumor has been small, sufficient has been available to show that it has a high extinction coefficient and a low diffusion current per unit of lipid, while normal muscle does not absorb at 260 mμ but does have a high diffusion current per unit of lipid. These observations suggest a structural alteration in this compound when normal mouse muscle becomes sarcomatous. On the other hand, the purified substance

TABLE 4

HALF-WAVE POTENTIALS IN VOLTS VERSUS S.C.E. AND THE ABSORPTION MAXIMUM OF THE REDUCIBLE MATERIAL FROM OTHER TISSUES

Material	pH 4.2–4.4 (volts)	pH 6.1–6.4 (volts)	pH 7.0–7.2 (volts)	Absorption maximum (mμ)
Liver	−1.33	−1.45	−1.50	260
Hepatoma	−1.30	−1.44	−1.51	260
Muscle	−1.34	−1.47	−1.51	
Rhabdomyosarcoma	−1.31	−1.43	−1.53	260

from liver and hepatoma have not only the same half-wave potentials as the total lipid material, but they also absorb maximally at 260 mμ between pH 3.8 and 9.2. These data indicate no difference between the substance in the liver and hepatoma, but further studies are necessary to establish this fact, since the substances obtained from the tissues mentioned above are either different polarographically or by their absorption in the ultraviolet, and yet they all appear somewhat similar with respect to physical appearance, solubility, hygroscopicity, and method of preparation. These facts would seem to indicate that these substances belong to one class of compounds.

DISCUSSION

The evidence presented in this report further demonstrates that a qualitative chemical change occurs in a substance extractable with alcohol and ether from epidermis undergoing carcinogenesis by methylcholanthrene. Alteration in this substance occurs abruptly, since the change is found in highly differentiated induced cancers, and remains the same as far as is discernible in transplantable tumors of different degrees of anaplasia (1, 2). This difference is expressed not only in the type of polarographic waves and in their half-wave potentials, but also in the absorption characteristics of the substance in the ultraviolet.

The tumor antigen of lipid character described by Waterman and Ebeling (5) does not seem to be the same as that from epidermis, since the preparation of the tumor antigen is different and it is a solid.

Furthermore, an alteration in a substance in muscle appears when it becomes sarcomatous, and present data indicate that this substance belongs to the same class of compounds as found in epidermis and carcinoma. This chemical change is manifested by a lack of absorption at 260 mμ. of the highly purified substance of normal muscle, while that of the muscle tumor absorbs strongly at 260 mμ between pH 3.8 and 9.2. The half-wave potentials of the substance in both tissues are about the same. On the other hand, the substance from normal mouse liver and hepatoma not only has the same half-wave potentials but also absorbs at 260 mμ from pH 3.8 to 9.2. Since such properties as physical appearance, solubility, hygroscopicity, and method of preparation of these substances are very similar for the tissues employed in these experiments, the conclusion seems warranted that they all belong to one class of compounds. Further investigations are now underway to determine, if possible, some of the functional groups of these substances.

SUMMARY

1. Evidence is presented to confirm the existence of a qualitative chemical change in a substance extractable with alcohol and ether from epidermis undergoing carcinogenesis. Alteration in the structure of the compound is manifested by a difference in the type of polarographic waves and in their half-wave potentials and also by a difference in absorption in the ultraviolet. There is also a dissimilarity between muscle and muscle tumor in that the purified substance from the latter absorbs at 260 mμ while that of the former does not. On the other hand, the substance from normal liver and hepatoma appears quite similar with respect to the half-wave potentials and absorption in the ultraviolet.

2. A method for the preparation of these reducible substances from various tissues is described, and some of the properties of the highly purified substances are given. Present data indi-

[2] This rhabdomyosarcoma was experimentally induced at the Institute for Cancer Research, Philadelphia, Pa., by Dr. E. Green, with a solution of methylcholanthrene injected intramuscularly in the left thigh. It was transplanted in July, 1945, into C3H mice.

cate that these substances belong to one class of compounds.

ADDENDUM

Recent experiments have demonstrated that the ratio, extinction coefficient at 260 mμ in mg. per milliliter: diffusion current in microamperes per milligram, of the purified substance from the hepatoma and rhabdomyosarcoma is nearly 3 times greater than the ratio of the purified substance from liver and muscle. The extinction coefficient of the substance from muscle was measured at pH 1.5 at 260 mμ. The extinction coefficient of the purified substance from the rhabdomyosarcoma was twice that of muscle while the diffusion current per milligram of substance of the former was nearly 50 per cent less than that of the latter. On the other hand the extinction coefficient of the purified substance from liver and hepatoma was nearly the same while the diffusion current per milligram of substance of the latter was 60 to 70 per cent less than that of the former.

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The Effects of a Transplanted Granulosa-Cell Tumor on Mice in Parabiosis*

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Growth of a transplanted granulosa-cell tumor in mice is associated with an increased blood volume and sinusoidal dilatation in the liver, spleen, and adrenal glands (4, 5, 8, 9, 10). The effect is not related to the estrogenic activity of the tumor (1, 6). In order to study the mechanism underlying these changes, parabiotic mice were used—one partner having a transplanted granulosa-cell tumor.

METHODS

Twelve pairs of parabiotic mice were studied. Fragments of a granulosa-cell tumor (18 C57) were transplanted into adult animals of the C57 black strain. This tumor originally arose in a castrated animal of the C57 strain that had a homologous transplant of an ovary into the pancreas (11); it is now in its fifth transfer generation. During the first and second transfer generation, mice given a preparation[1] containing progesterone had an increased frequency of growth of the tumor. The tumor requires no extrinsic hormonal supplement.

The transplants were made subcutaneously using a #15 gauge trocar. As soon as a small nodule was palpable at the site of the transplant, the animal was united in parabiosis to a nontumor-bearing litter-mate. The technic used was that of Sauerbruch and Heyde (13), as modified by Bunster and Meyer (3).

Eleven of the twelve pairs of mice were castrated 5–7 days before being united in parabiosis, and vaginal smears were made at intervals during the course of the experiment. All animals were kept on a standard diet and water *ad libitum*.

* This investigation was supported by a research grant from the U.S. Public Health Service.

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[1] An unrefined concentrate containing approximately 200–250 I.U. progesterone/cc; obtained from the Glidden Company through the courtesy of A. E. Engstrom.

Received for publication, January 21, 1950.

OBSERVATIONS

Nontumor-bearing animals were in parabiotic union with tumor-bearing animals from 11 to 110 days before either dying or being sacrificed. Following parabiosis, only those animals that gained weight and showed a normal amount of activity were studied.

At autopsy the animals with tumors had enlarged livers and spleens (Table 1). Grossly, the adrenal glands of these animals were enlarged and congested, and the hearts were dilated but had not increased in weight. None of these changes were observed in the nontumor-bearing partners. Vaginal smears of the castrate tumor-bearing females with large tumors indicated the presence of an estrogenic substance, while the smears of the castrate nontumor-bearing parabionts were all of a diestrous type.

Histological examination of the livers of animals with tumors revealed sinusoidal dilatation varying from slight to extreme dilatation, with isolated cords of liver cells between large dilated sinuses. No sinusoidal dilatation was observed in the livers of the nontumor-bearing partners (Figs. 1 and 2).

The spleens of the tumor-bearing animals showed greater vascular engorgement than the spleens of the nontumor parabionts. There was also dilatation of the sinusoids at the corticomedullary junction of the adrenal glands of the tumor-bearing animals (Figs. 3 and 4).

The uteri of the castrate tumor females were enlarged and showed the effects of estrogenic stimulation, while the uteri of the castrate nontumor-bearing female partners were small and showed the typical changes of castration (Figs. 5 and 6).

DISCUSSION

In this series, animals with tumors weighing 1 gm. or more had sinusoidal dilatation in the liver, spleen, and adrenal glands, yet these changes were not observed in the nontumor-bearing partners united in parabiosis.

While blood volume studies were not performed in this experiment, the sinusoidal dilatation in the tumor animals was secondary to an increased blood volume (9). The observation that the nontumor-bearing parabiont does not develop any of the hypervolemic effects when united in parabiosis to an animal bearing a granulosa-cell tumor for periods up to 110 days may be due to the fact that the agent responsible for the hypervolemia is not produced in sufficient concentration to cross over to the normal animal, or it may be inactivated more efficiently by the normal animal. The possibility of a concomitant infection associated with this tumor as an explanation of the hypervolemic changes does not seem tenable, as the presence of vascular anastomoses between parabiotic animals has been demonstrated. When the dye, brilliant-red, is injected intravenously into one parabiotic rat, it is present in equal concentrations in both animals in about 6 hours (12); and rat erythrocytes injected intravenously into one parabiotic mouse are present in the blood of the nontreated partner in 20 minutes, and large numbers are present 120 minutes after the injection (7). If the hypervolemic effects were due to an infectious agent of the nature of a virus associated with this tumor, then it is likely that these effects would occur in the nontumor-bearing partner.

The estrogenic substance produced by the tumor is in quantities too small to cross over into the normal animal, or it is quickly inactivated, for its effects are not observed in the nontumor-bearing parabiont. When estradiol is injected into one parabiotic rat, the concentration of estradiol must be increased approximately 40 times before its effects will be observed in a parabiont twin (2).

TABLE 1

AUTOPSY DATA ON TUMOR AND NONTUMOR MICE IN PARABIOSIS*

Animal number	Days of parabiotic union	Heart weight (mg.)	Liver weight (gm.)	Spleen weight (mg.)	Tumor weight (gm.)	Degree of liver sinusoidal dilatation†
169♂	25	160	1.17	120	.160	−
184♂		290	1.14	180		−
80♀	54	170	1.74	170	1.87	++++
39♀		230	1.48	350		−
37♀	32	210	1.49	330	2.12	+++
120♀		190	.99	90		−
128♂	42	250	2.56	420	2.85	+++
22♀		150	1.12	340		−
27♀	30	190	1.90	260	3.22	+++
25♀		110	1.25	170		−
22♀	64	150	2.46	320	3.99	++++
23♂		140	1.08	300		−

* Only animals sacrificed included in table.

† − to +: beginning sinusoidal dilatation.
+ to ++: early sinusoidal dilatation.
++ to +++: moderate sinusoidal dilatation.
+++ to ++++: extreme sinusoidal dilatation.

SUMMARY

1. C57 mice, when united in parabiosis for periods up to 110 days to litter-mates bearing a transplanted granulosa-cell tumor, do not develop the hypervolemic effects seen in the tumor-bearing animals.

2. It is unlikely that the hypervolemia associated with mice bearing a granulosa-cell tumor is due to an infectious agent.

3. The estrogenic-like substance produced by a transplanted granulosa-cell tumor in one mouse of a parabiotic pair is not sufficient to stimulate a castrate parabiont twin.

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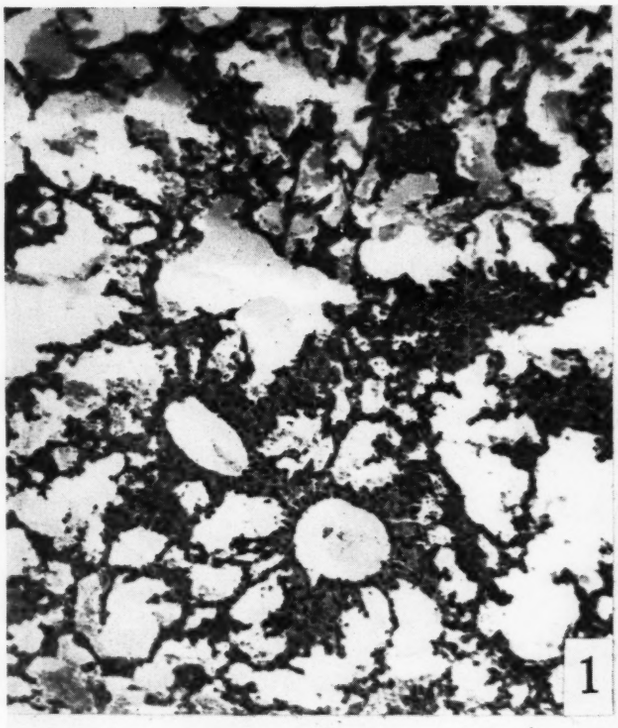

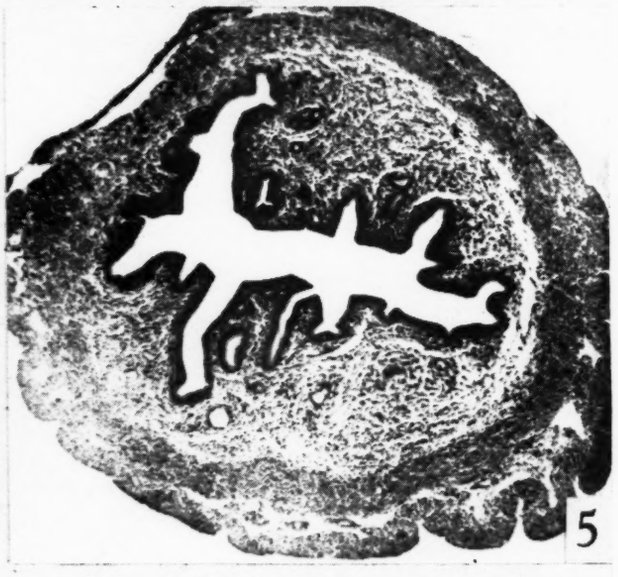

FIGS. 1–6

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FIG. 1.—Section of liver of tumor-bearing animal No. 80 C57 showing sinusoidal dilatation. Mag., 32 mm. objective and 8 × ocular.

FIG. 2.—Section of liver of nontumor-bearing animal No. 39 C57 parabiosed to No. 80 C57 for 54 days. Mag., 32 mm. objective and 8 × ocular.

FIG. 3.—Section of adrenal gland of tumor-bearing animal No. 80 C57. Mag., 32 mm. objective and 8 × ocular.

FIG. 4.—Section of adrenal gland of nontumor-bearing animal No. 39 C57. Mag., 32 mm. objective and 8 × ocular.

FIG. 5.—Section of uterus of tumor-bearing castrate animal No. 80 C57. Mag., 32 mm. objective and 8 × ocular.

FIG. 6.—Section of uterus of nontumor-bearing castrate animal No. 39 C57. Mag., 32 mm. objective and 8 × ocular.

Differential Radiosensitivity of Haploid and Diploid Prepupae and Pupae of Habrobracon*

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INTRODUCTION

Living cells and organisms, when in various physiological states, differ in susceptibility to x-radiation. It has been shown that actively dividing cells are more sensitive than interphasic cells, while cells in metaphase appear to be more sensitive than those in early prophase. It is also recognized that different tissues show differing radiosensitivity. This is especially manifested among various types of neoplasms. The underlying reasons for this are not known.

Numerous investigations have shown that cell death following radiation may be due to chromosome breakage with resultant lethal arrangements or loss of fragments. Little is known concerning the relation of radiation sensitivity to the number of chromosome sets. It might be expected that such exists. A lethal type of change in a chromosome may still permit the normal functioning of its homologue in a diploid organism. Such a change in a haploid organism would lead to death.

Müntzing (4), studying the effects of x-radiation on the growth and development of diploid and tetraploid barley treated as seeds, found that tetraploids are much more resistant to x-rays than diploids. The effects of x-rays on haploid and diploid forms of the yeast *Saccharomyces cerevisiae* were investigated by Latarjet and Ephrussi (2). They showed by survival curves that the haploids are more sensitive than the diploids. Lamy and Muller (1), however, x-rayed diploid and triploid Drosophila embryos and found no difference in susceptibility. It was concluded by these authors and by Muller (3) that the lethal effect of x-rays on the embryos was of a nongenetic ("physiological") nature. Whiting and Bostian (7) found that the haploid male larvae of the wasp Habrobracon were more sensitive to x-radiation than were the diploid female larvae. This may be interpreted in terms of a sex difference, rather than a difference in the number of chromosome sets. Since, in Habrobracon, individuals of the same sex which differ in the number of chromosome sets may be obtained, it is possible to determine with this organism which of these interpretations is valid.

The purpose of this investigation was to test the lethal action of x-rays upon haploid male, diploid male, and diploid female prepupae and pupae. The data can then be used to determine whether there is a correlation between radiosensitivity and the number of chromosome sets irrespective of sex.

MATERIALS AND METHODS

The wasp Habrobracon is parasitic upon the larvae of the Mediterranean Flour Moth Ephestia. Female wasps normally sting these larvae, paralyze them and lay eggs upon them. The wasp larvae hatching from these eggs feed upon the body juices of the host and when feeding is completed leave and spin cocoons. Metamorphosis takes place within the cocoons from which the adults eclose. At 30° C., the entire life cycle is from 9 to 10 days. The larvae hatch from the eggs after 30 hours and start to spin cocoons after 70 hours. The period from the start of cocoon spinning to egestion of a meconium (70 to 108 hours after oviposition) is known as the prepupal period. The period after meconium formation and before eclosion is known as the pupal period.

Two Habrobracon females were placed in each of several Stender dishes on paper discs with five Ephestia larvae and were allowed to lay eggs for 12 hours. Transfers of females to new hosts for subsequent 12-hour intervals were then made. The Habrobracon cocoons become attached to the paper discs and are therefore easy to handle. The discs containing the cocoons were then removed from the Stender dishes and irradiated. The prepupae and pupae were x-rayed at various ages within the period from 72 to 120 hours and were observed until 15 days from the time of egg laying. The number of adults eclosing during this period was recorded. At the end of this time, the cocoons still containing individuals unable to eclose were

* This project was supported by a grant from the Faculty Research Committee of the University of Delaware.

Received for publication, January 30, 1950.

cut open and observations of structural changes, sex type, and stage of development were made.

Haploid males arise from unfertilized eggs while diploid males and diploid females arise from fertilized eggs. When females are crossed to unrelated males, two classes of offspring, haploid males and diploid females, are obtained. However, when females are crossed to related males, diploid males also occur.

an inherent filter equivalent to 0.2 mm. copper. The output intensity was 2,860 r per minute.

EXPERIMENTAL

Habrobracon cultures consisting entirely of haploid males were obtained from unmated mothers. These haploids were x-rayed in their cocoons at ages from 72 to 120 hours. Preliminary doses ranging from 1,000 r to 6,000 r were used in

TABLE 1

ECLOSION RATIOS OF HAPLOID MALES AND DIPLOID FEMALES

Dose (roentgens)	Age at x-radiation (hours)	Number x-rayed	Number unclassified	Eclosion ratios n ♂	Eclosion ratios 2 n ♀	Per cent eclosion n ♂	Per cent eclosion 2 n ♀
0		322	0	293/322		91.0±0.8	
6,000	108–20	120	0	27/120		22.5±3.7	
5,000	96–108	75	0	0/75		0.0±0.0	
4,000	84–96	126	0	1/126		0.8±0.8	
4,000	72–84	116	0	5/116		4.3±1.8	
0		116	0	68/68	48/48	100.0±0.0	100.0±0.0
6,000	108–20	210	29	14/99	67/82	14.1±3.1	81.7±4.3
5,000	96–108	219	15	2/89	68/115	2.2±1.5	59.1±4.6
4,000	84–96	423	30	9/210	98/183	4.3±1.4	53.6±3.7
4,000	72–84	353	55	1/157	37/141	0.6±0.5	26.2±3.7

TABLE 2

ECLOSION RATIOS OF HAPLOID MALES, DIPLOID FEMALES, AND DIPLOID MALES

Dose (roentgens)	Age at x-radiation (hours)	Number x-rayed	Number unclassified	Eclosion ratios n ♂	Eclosion ratios 2 n ♀	Dose 2 n ♂
0		308	9	161/162	118/120	17/17
4,000	108–20	52	8	18/23	20/20	1/1
5,000		283	39	53/137	88/103	4/4
6,000		266	39	24/116	76/100	5/11
7,000		113	11	2/64	24/38	0/0
8,000		43	11	0/13	8/18	1/1
9,000		9	1	0/3	2/5	0/0
4,000	96–108	141	17	26/76	39/44	2/4
5,000		411	73	9/164	87/161	9/13
6,000		193	35	0/103	17/54	0/1
7,000		42	6	0/16	4/20	0/0
8,000		18	1	0/8	1/9	0/0
3,000	84–96	95	9	37/56	29/29	1/1
4,000		399	63	7/187	84/138	3/11
5,000		87	38	0/22	1/27	0/0
6,000		45	29	0/3	0/13	0/0
3,000	72–84	59	3	13/30	20/23	2/3
4,000		369	109	0/157	41/100	2/3
5,000		39	25	0/7	0/6	0/1
6,000		30	30	0/0	0/0	0/0
Summary for x-rayed individuals:		2,694	547	189/1185 15.9±1.1 per cent	541/908 59.6±1.6 per cent	30/54 55.6±6.7 per cent

Data presented in Table 1 were from individuals x-rayed at the Biochemical Research Foundation. The machine was run at 250 kv. and 15 ma. The inherent filter was equivalent to 3 mm. of aluminum. The prepupae and pupae were placed 23 cm. from the target. The dosage was 792 r per minute. Data collected for Table 2 were from individuals x-rayed at the Marine Biological Laboratory. The conditions of radiation were as follows: the secondary voltage was 182 kv., and the tube current in each x-ray tube was 25 ma. There was

order to select doses sufficiently high to prevent eclosion of most of the haploids. The doses selected and the eclosion ratios obtained for each age group are summarized in Table 1. A sharp rise in resistance to radiation was noted for individuals treated within the 108–20 hour period (pupal period). The pupae, as shown by the eclosion ratios, are much more resistant than are the prepupae. The untreated controls bred concurrently showed an eclosion ratio of 91.0 ± 0.8 per cent. This shows that the stock used had a high viability. The same doses

selected for the haploid males were used to compare the radiosensitivity of the haploid males with the diploid females.

Cultures containing only haploid males and diploid females were obtained by crossing stock #25 females to the distantly related males from stock #1. From this cross all of the unfertilized eggs normally result in haploid males, while all of the fertilized eggs become diploid females. Comparison of eclosion ratios of haploid males with those of diploid females for each age group shows that the females are much more radio-resistant (Table 1).

Eclosion ratios for the untreated controls show that there is no differential mortality between haploid males and diploid females (Table 1). In obtaining eclosion ratios it was not possible to determine the sex of some of the individuals. These are listed as unclassified and represent those which had not metamorphosed sufficiently to have developed genitalia. The unclassified individuals in no way upset the striking differences in viability between the haploid males and diploid females. Even if these were classified as diploid females the differences in eclosion ratios between the groups would still be significant.

Cultures containing all three sex types (haploid males, diploid males, and diploid females) were obtained by crossing cantaloupe eye-colored females from stock #25 to black-eyed males from the same stock. Eye color is used as a marker to distinguish between the haploid males and the diploid males. All of the haploid males thus have cantaloupe eye color while all of the diploid males have black eye color. Diploid males do not occur as frequently as do haploid males or diploid females. This is shown for the untreated controls (Table 2), where 162 haploid males, 120 diploid females, and 17 diploid males were obtained. Comparison of eclosion ratios among these three types shows that no differential mortality occurs after the prepupal stage.

The results, summarized in Table 2, show the eclosion ratios for different doses and ages for the three sex types. Presentation of these ratios permits comparison of diploid male viability with that of the haploid male and diploid female. Too few diploid males were obtained in each group to express these data in terms of eclosion percentages. The data were, therefore, pooled, and the per cent eclosion is expressed for the total number of offspring for each sex type.

The percentage eclosion for haploid males is significantly lower than those obtained for the diploid males and diploid females. Comparison of the diploid male eclosion ratios with those of the diploid females shows no significant difference. The difference in radiosensitivity among these groups is, therefore, probably due to the number of sets of chromosomes rather than to the sex difference.

The prepupae and pupae irradiated at the Marine Biological Laboratory were given x-rays at the rate of 2,860 r per minute, while those at the Biochemical Research Foundation were given 792 r per minute. Comparison, therefore, can be made between the eclosion ratios of individuals receiving the same total x-ray dose at different intensities. The pooled eclosion percentage for haploid males given 4,000–6,000 r at 792 per minute (Table 1) was 4.7 $\pm$ 0.9 per cent while for comparable haploid males treated at 2,860 r per minute (Table 2), it was 6.4 $\pm$ 1.0 per cent. Similarly, the eclosion percentages for females treated at 792 r per minute and 2,860 r per minute was 51.8 $\pm$ 2.2 per cent and 57.7 $\pm$ 2.2 per cent, respectively. There is no significant difference between the comparable groups given the same total dose, but at different intensities.

Many of the adults eclosing from cocoons following treatment exhibited morphological abnormalities. The degree of abnormality was greater for higher doses. It appears that the antennal, wing, and leg anlagen were affected more than other developing structures. In most cases, the effect was more pronounced upon the wings and legs than upon the antennae. Some incompletely metamorphosed individuals showed that these appendages had been arrested in development, while the abdomen and head continued to develop into complete and normal adult structures. Wing abnormalities ranged from wrinkling to "wing stubs" due, perhaps, to the inability of these structures to expand properly. Leg abnormalities were localized primarily in the tarsi and prevented normal locomotion. Antennal abnormalities were shown by the fusion of flagellar segments. Examination of those adults eclosing showed that the degree of abnormality was greater for the haploids than for the diploids.

Examination of individuals left in cocoons revealed adults that were too weak to eclose and pupae in various stages of arrested development. Almost all these pupae were still alive when removed from the cocoons. On the average, the diploid individuals had advanced to a later stage of development than had the haploids.

DISCUSSION

Lamy and Muller (1) attempted to distinguish between the genetic and nongenetic effects of the lethal action of x-rays by studying Drosophila embryos differing in the number of sets of chromosomes. In comparing radiation effects upon dip-

loids and triploids they found no difference in the mortality rates for these two groups. Doses used and data obtained are not given. They conclude, therefore, that the lethal effect of x-rays upon Drosophila embryos is of a nongenetic, "physiological" nature. The results reported here for Habrobracon differ from those reported by Lamy and Muller. Comparison of eclosion ratios among haploid males, diploid males, and diploid females shows that diploids, irrespective of sex, are more resistant than haploids. If the susceptibility of organisms differing in the number of chromosome sets is to be accepted as the criterion for separating genetic and nongenetic effects of radiation, it may be suggested that in Habrobracon the lethal action of x-rays is due to an effect upon the chromosomes (genetic effect). The observations of Whiting and Bostian (7) on haploid male and diploid female Habrobracon larvae, of Müntzing (4) on diploid and tetraploid barley, and of Latarjet and Ephrussi (2) on haploid and diploid yeasts are in agreement with these data in that they show that the organisms with the greater number of sets of chromosomes are more resistant to x-radiation.

Lamy and Muller (1) report that there is no orderly difference in degree of structural abnormality between diploid and triploid Drosophila. They, therefore, conclude that "physiological" rather than genetic changes were the cause of these abnormalities. The present study shows that adult haploid Habrobracon show greater degrees of structural abnormality than do diploids and that diploids which fail to eclose are more advanced in their development than are haploids. Also, Müntzing (4) finds that in barley the incidence of germination and the decrease in vigor of the resulting plants following x-radiation is less for tetraploids than for diploids.

In order to explain the seemingly conflicting observations, it must be said that different organisms are involved and, therefore, results peculiar to each organism are certainly possible. Studies of radiation effects on other organisms would be required before a generalization can be made. It is also feasible that the triploid to diploid chromosome ration (3 to 2) is not as great as the diploid to haploid (2 to 1) or the tetraploid to diploid (4 to 2) and is, therefore, not as sensitive for testing the differential lethal action of x-rays. Also, since Lamy and Muller do not report their x-ray dose, it is possible that the optimum dose necessary to show a difference was not used. Studies, not reported here, have shown that doses of 2,000 and 3,000 r, when used for Habrobracon pupae of certain ages, have produced no differential response. The junior author has shown (unpublished) that the susceptibility of embryos to the lethal action of x-rays may vary for dosages from 400 to 30,000 r, depending upon the age.

A possible explanation for the greater resistance of diploid Habrobracon prepupae and pupae than for the haploids is that the x-rays have caused chromosome injury, particularly the loss of chromosome fragments. In diploids the loss of a part of any one chromosome would be compensated for by the presence of an identical part in the homologous chromosome. In the haploids, since each chromosome is represented singly, the loss of a chromosome fragment would be lethal.

Further indication that the x-ray effect may be of a chromosomal nature is shown by comparing eclosion ratios of individuals receiving the same total dose but at different intensities. The data presented show that there is no significant difference between individuals given the same total dose at 792 r per minute and at 2,860 r per minute. There is, therefore, an independence of response to change in intensity.

It does not follow from these conclusions that there can be no cytoplasmic injury due to x-radiation. Whiting (6) has been able to differentiate cytoplasmic from chromosomal injury following the x-radiation of Habrobracon eggs. She has shown that with doses up to 15,000 r death is due to chromosomal injury. Above 15,000 r, cytoplasmic injury occurs and increases up to about 54,000 r, which is the lethal dose for the cytoplasm. It seems, therefore, that the relatively small doses used here would preclude the probability that death is due to cytoplasmic (nongenetic) injury and favor the hypothesis that it is due to lethal types of chromosomal change.

The data indicate that a correlation exists between the number of chromosome sets and radiosensitivity. However, it is well known that other factors reflecting the physiological condition of the organism are also involved. These data show that pupae are more resistant than prepupae. Smith (5) found that the difference in a single gene was responsible for increase in radiosensitivity of dormant seeds of Triticum monococcum. Thus, the number of chromosome sets represents one of many factors. Perhaps the general condition of the cell as determined by the chromosome number is to be considered.

SUMMARY

1. Habrobracon prepupae and pupae were exposed to x-radiation, and the lethal effects of the radiation were studied.

2. Eclosion ratios show that x-rays have a greater lethal effect upon haploid males than upon

diploid males or diploid females. Diploid males and diploid females are equally susceptible to the lethal effects of x-radiation. Prepupae are more sensitive than pupae.

3. No significant difference in eclosion ratios was obtained for adults given the same total dose but at different intensities. The response, therefore, seems to be independent of intensity.

4. Comparison of adults developing from irradiated prepupae and pupae shows that a greater number of haploid males show structural malformations than do diploids irrespective of sex and that the haploids show these malformations to a greater degree. Comparison of individuals unable to emerge from cocoons shows that the diploids are in general more advanced in their development than are the haploids.

5. The data show that diploids are more resistant than haploids to the lethal action of x-rays, suggesting that the number of chromosome sets is a factor in determining the radiosensitivity of cells.

ACKNOWLEDGMENT

The authors wish to thank Dr. Ellice McDonald for allowing them use of x-ray equipment at the Biochemical Research Foundation, and to Drs. P. W. and A. R. Whiting for their suggestions and criticisms during the preparation of this manuscript.

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Citric Acid Content of Normal and Tumor Tissues *in Vivo* following Injection of Fluoroacetate*

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Previous investigations have indicated that tumor tissues are deficient in enzymes which oxidize pyruvate via the Krebs citric acid cycle. Elliot and Grieg (2) concluded that tumor slices converted little or no pyruvate to succinate. They observed the disappearance of keto-acids, but not acid groups, when oxalacetate or pyruvate was added to the media. The observation that little succinate accumulated in the presence of pyruvate and malonate must be discounted, because at that time the role of oxalacetate was not fully apparent, and none was added to the reaction mixture. Whole homogenate systems were developed in this laboratory (6) for the study of the Krebs oxidation cycle, and, when whole tumor homogenates were studied with oxalacetate added to the medium (8), oxygen uptake was almost completely lacking in comparison with several normal tissues. Potter, Pardee, and Lyle (9) found that citrate formation in homogenates of tumor was very low compared to that in normal tissues, and utilization of oxalacetate was equally small. In experiments with glycolyzing tumor homogenates, Potter and LePage (7) observed that tumor homogenates, even while maintaining their ATP through glycolysis, could not oxidize oxalacetic acid via the Krebs cycle, since essentially no citric acid was accumulated under anaerobic conditions. The work of Buffa and Peters (1) offered a means for testing the ability of tumor to produce citrate *in vivo*, since oxidation of citric acid is prevented and the compound accumulates following injections of sodium fluoroacetate.

MATERIALS AND METHODS

Adult rats obtained from the Holtzman Rat Company were injected intraperitoneally with 5 mg. of sodium fluoroacetate[1] per kilogram body weight (1). A single sample of fluoroacetate was used throughout this experiment, since it was shown that the effects were substantially the same as those produced by Buffa and Peters (1) in the three tissues examined in both laboratories. While the dose administered is lethal within 16–24 hours, significant metabolic effects as well as toxic signs are noted in the relatively short period of ½–2 hours. According to the technic of Buffa and Peters (1), the rats were generally decapitated 1 hour after the injection. The following tissues were then dissected out and dropped into ice-cold isotonic KCl: brain, heart, lung, thymus, liver, kidney, spleen, pancreas, testis, and muscle. In addition, a blood sample was taken from the severed neck vessels, and coagulation was prevented by the addition of potassium oxalate. The tissues were weighed, placed in an equal volume of isotonic KCl, and homogenized before and after the addition of a volume of 20 per cent trichloracetic acid (TCA) equal to the combined volumes of tissue and isotonic KCl. Citrate content in micrograms per gram of tissue was determined on the TCA filtrate by the pentabromoacetone method, as modified by Natelson, Lugovoy, and Pincus (5). Certain modifications are of interest: in the citrate analysis, the effect of excess hydrogen peroxide in the recommended concentration (6 per cent) was studied, and it was found that each extra drop beyond the amount necessary to decolorize the permanganate increased the E value 3–4 per cent; to minimize this potential source of error, the hydrogen peroxide was diluted to one-half the recommended concentration, and considerable caution was exercised in its addition to the solutions. The stability of the refrigerated TCA filtrates over a period of time was a matter of practical interest; the citrate content of groups of these filtrates was determined the same day on which the samples were obtained and also 1–4 days later. In no case was the mean difference between the readings greater than 4 per cent, and, since the accuracy of the method is close to this value, it was concluded that the citrate content of cold TCA filtrates does not change significantly in a 4-day period. In most

* This work was supported by a grant from the U.S. Public Health Service and by the Jonathan Bowman Fund for Cancer Research.

[1] This was kindly supplied by Dr. K. P. Dubois.

Received for publication, February 2, 1950.

cases, the samples were analyzed within 24–48 hours. The size of the TCA aliquot depended upon the citrate content of the tissue; for normal, uninjected animals, 2.0 ml. of TCA filtrate were used, when available, since the citrate content of the tissues was generally quite small. For the injected animals, the following aliquots were selected: liver, muscle, testis, tumors, and blood, 2.0 ml.; brain, thymus, lung, and pancreas, 1.0 ml.; heart and spleen, 0.5 ml.; kidney, 0.3 ml. In the case of lung, 0-58, 0-46, and 0-48 in heart, brain, and kidney, respectively. In the injected animals, they found a range of 558–810, 118–271, and 854–1220 in the same three tissues. Large increases in citrate content in the fluoroacetate-injected animals are noted in the spleen, thymus, lung, and pancreas, in addition to increases in the heart, brain, and kidney, which had been reported earlier (1). Blood, muscle, and testis showed small increments in citrate content, while the liver showed no increase.

TABLE 1

EFFECT OF FLUOROACETATE ON THE CITRATE CONTENT OF NORMAL TISSUES IN NORMAL AND TUMOR-BEARING RATS

Values are presented in micrograms per gram wet weight of tissue in each case.

Tissue	Normal animals			Normal animals 1 hour after injection			Tumor-bearing animals 1 hour after injection		
	No. of samples	Average	Range	No. of samples	Average	Range	No. of samples	Average	Range
Brain	5	57	35 – 65	5	212	169– 241	5	160	134– 234
Heart	5	49	24 – 73	5	632	525– 697	5	448	289– 638
Lung	5	75	40 –114	5	285	205– 422	5	206	133– 295
Thymus	5	55	24 – 79	5	525	297– 713	5	275	230– 337
Liver	5	47	20 – 86	5	50	39– 75	5	39	6– 77
Kidney	5	56	16 –123	5	1,029	735–1,542	5	714	445–1,039
Spleen	5	59	28 – 87	5	652	502– 791	5	514	306– 754
Testis	5	73	58 –113	5	114	60– 173	4	79	49– 119
Blood	5	54	35 –114	5	74	42– 109	5	79	48– 102
Muscle	4	31	25 – 38	5	54	46– 69	2	42	41– 42
Pancreas	3	53	29 – 78	2	276	197– 372			

TABLE 2

EFFECT OF FLUOROACETATE ON THE CITRATE CONTENT OF TUMORS AND BLOOD OF TUMOR-BEARING ANIMALS

Values are presented in micrograms per gram wet weight of tissue; injected animals' tissues collected 1 hour after injection with fluoroacetate.

Tumor	Uninjected tumor-bearing animals			Injected tumor-bearing animals		
	No. of samples	Average	Range	No. of samples	Average	Range
Walker 256 carcinoma	4	49	38– 61	4	42	31– 52
Blood	4	59	53– 62	3	63	53– 84
Flexner-Jobling carcinoma	4	121	92–144	4	90	61–119
Blood	4	74	48–102	3	90	84–102
Jensen sarcoma	4	85	53–141	3	66	40–116
Hepatoma, primary	2	95	80–110	2	60	52– 67

it was essential that the tissue was not waterlogged with absorbed KCl solution, since this produced large errors in the wet-weight measurement. With the exception of the primary hepatomas studied, the tumors generally weighed less than 1 gm. and were 8–12 days post-transplantation; no gross evidences of necrosis or degeneration were noted.

RESULTS

The effect of fluoroacetate on the citrate content of various tissues 1 hour after injection, as compared with the citrate content of the normal tissues is indicated in Table 1. These data confirm the results of Buffa and Peters (1) with respect to the kidney, heart, and brain; they found that the normal level of citrate in micrograms per gram was

Citrate accumulation in the normal organs of tumor-bearing rats was investigated in three rats bearing Flexner-Jobling carcinomas and in two rats bearing Walker 256 carcinomas. The results are presented in Table 1, for comparison with data on organs from nontumor-bearing animals. There is a general preponderance of higher values for animals without tumors. It is possible that the tumor not only has a deficiency in enzymes concerned with citrate formation but also may exert a depressant action on the citrate accumulation of other tissues in the tumor-bearing animal. However, the data are not sufficient to establish the point, and it is incidental to the main topic of this report.

Four tumor types were studied: the Flexner-

Jobling carcinoma, Walker 256 carcinoma, Jensen sarcoma, and primary hepatomas.[2] Tumor-bearing rats were injected with fluoroacetate and sacrificed 1 hour after the injection; for comparison, uninjected tumor-bearing rats were also sacrificed, and the tumors were analyzed for citrate. The results (Table 2) show that no increases in the citrate level of the tumors of the injected animals occurred. While it is true that, in all cases, the tumors of injected animals had decreased citrate levels compared with those in the uninjected animals, the significance of this observation cannot be evaluated at this time, because of the ranges of citrate content. It can be seen that a close similarity exists between the blood levels of citrate and the level of citrate in the tumor; in general, the blood had slightly more citrate per gram than did the tumors.

The rate of citrate accumulation was studied with particular reference to several organs in which the citrate content increases greatly. Three rats were observed ½ hour, and three others 2 hours, after injection with fluoroacetate, in the same manner as noted for the 1-hour rats. The values for citrate content were averaged for the group at ½ hour, and also for the group at 2 hours, and compared with the averages for the normals and 1-hour animals (Table 3). It is apparent that the increase in citrate content was maximal during the first hour; while the spleen showed some increase in citrate content in the second hour, the thymus and lung showed slight increases, and the heart, kidney, and brain showed essentially no change. For this reason, the studies on tumor-bearing rats were made 1 hour after the injection of fluoroacetate. This was also the time interval used by Buffa and Peters (1).

TABLE 3

CITRATE CONTENT OF VARIOUS TISSUES AT INTERVALS AFTER FLUOROACETATE INJECTIONS

Average citrate data from three rats sacrificed 30 minutes after injection and three rats sacrificed 2 hours after injection are presented along with average data for normal tissues and tissues of rats sacrificed 1 hour after injection with fluoroacetate. The rate of accumulation decreases markedly after 1 hour.

TISSUE	INTERVAL			
	0 hours	½ hour	1 hour	2 hours
Brain	57	94	212	204
Heart	49	306	632	690
Lung	75	192	285	311
Thymus	55	189	525	552
Spleen	59	366	652	792
Kidney	56	477	1,029	1,002
Blood	53	41	74	91

[2] Induced by feeding diets containing 4-dimethylaminoazobenzene. We are indebted to Dr. E. C. Miller and Dr. J. A. Miller for these tumors, and to Dr. G. A. LePage for the transplantable tumors.

To test the protective ability of sodium acetate against the effect of sodium fluoroacetate, an experiment was performed in which varying concentrations of acetate were injected intraperitoneally into normal rats, either simultaneously with the fluoroacetate or ½ hour later; and the rats were sacrificed 1 hour after the fluoroacetate injection. The kidney, spleen, and heart were tested for citrate content, and the results are noted in Table 4.

TABLE 4

EFFECT OF ACETATE ON CITRATE ACCUMULATION IN THE ANIMAL INJECTED WITH FLUOROACETATE

The animals were sacrificed 1 hour after injection with fluoroacetate; t_0 refers to time of injection of fluoroacetate; t_{30}, 30 minutes after fluoroacetate injection.

Amount acetate injected	Time of injection	Heart	Kidney	Spleen
50 mg.	t_0	619	808	422
5 mg.	t_0	386	621	512
1 mg.	t_0	563	1,253	718
5 mg.	t_{30}	422	1,048	606

From these data it can be inferred that acetate at these levels provided no significant protection, as no consistent diminution of citrate content in these organs, as compared with the normal injected rats, is apparent—either from differences within the series or by direct comparison with the data in Table 1.

DISCUSSION

Evidence from whole animal experiments is presented to support earlier *in vitro* indications that tumor tissues are deficient in enzymes which oxidize pyruvate via the Krebs citric acid cycle. It is relevant to consider the rate of pyruvate oxidation in terms of the rate of pyruvate *formation* in tumor tissues. It has been shown with homogenates that these tissues have a capacity for producing pyruvate at the rate of approximately ½ μM/mg wet weight/hour (3); this is equivalent to 3 μM pyruvate/mg dry weight, and if this pyruvate were oxidized to CO_2 as fast as it can be formed, the resulting Q_{O_2} would be 201.8, inasmuch as combustion of triose phosphate or lactate requires 3 μM oxygen/μM. On the basis of the glycolytic rates of tumor slices in the presence of glucose, a Q_{O_2} of 80.2 for combustion of pyruvate resulting from glucose breakdown in slices would be required. The actual Q_{O_2} of tumor slices is between 7 and 10, and the pyruvate which is not oxidized is converted to lactate. While the substrates that contribute to the endogenous Q_{O_2} have never been specified, it seems possible that there may be a partial oxidation of pyruvate at a rate which may exceed the over-all rate for the Krebs cycle as a whole. Rates corresponding to a Q_{O_2} of less than

one for the cycle have not been excluded, but, at this level, isotope technics are required for further information. The *in vivo* studies reported above are of considerable interest in that they appear to provide a very sensitive indication of the extent of conversion of pyruvate to citrate, and no accumulation of citrate could be found in tumor tissues. Further studies will be required to define the nature and extent of the partial oxidation of pyruvate that is implied by the existing data on the Q_{O_2} of tumor slices, and isotope experiments on tumor slices are in progress.

The interpretation of the data on citrate accumulation in the presence of fluoroacetate *in vivo* must consider the following possible factors: (*a*) the activity of the enzymes of the Krebs cycle, (*b*) the availability of oxalacetate from sources other than citrate as the oxidation of the latter compound becomes blocked, (*c*) the ability of the tissue to "activate" fluoroacetate (4), (*d*) the outward diffusion of the citrate from the tissues to the blood, and (*e*) the presence of alternative pathways for the disposition of acetate and fluoroacetate.

The prominence of the alternative pathway to acetoacetate in liver is believed to account for the failure of that organ to accumulate citrate. Separate *in vitro* experiments will be reported to show that liver is capable of accumulating large amounts of citrate but that fluoroacetate converts a citrate-producing system to an acetoacetate-producing system. Variations in the ability to activate acetate and fluoroacetate (point 3) may make the effective dose for citrate accumulation differ from organ to organ and from the effective lethal dose, and experiments with tenfold the lethal dose have resulted in increased citrate production in certain normal tissues but not in tumor tissues.

The rate of citrate loss from the tissues (point 4) is apparently not great enough to result in citrate accumulation in the blood. The differences among the various tissues, and the fact that very high tissue levels can be attained without raising the blood level, are strong evidence that the tissue citrate equilibrates very slowly with the blood. Preliminary experiments with the tenfold lethal dose indicate that in some cases this barrier may break down, and the possibility that tumor tissues release citrate while normal tissues retain it at the dosages employed in the present study must be borne in mind.

In spite of these limitations in the interpretation of the data, the results may be regarded as strong evidence that the ability of tumor tissues to form citrate is very low in comparison with a wide variety of normal tissues.

SUMMARY

1. Marked accumulations of citrate occurred in most normal rat tissues within 1 hour after the injection of a lethal dose of fluoroacetate (5 mg/kg). Essentially no further increase occurred after the first hour.

2. Under the same conditions, no citrate accumulation occurred in tumor tissues or in normal liver. The lack of citrate accumulation in the liver may result from the presence of an alternative pathway of metabolism that leads to acetoacetate; but, in the case of the tumor tissue, the data are regarded as further evidence for a decreased ability to effect the Krebs condensation.

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Factors Affecting the Number of Tumor Metastases
Experiments with a Transplantable Mouse Tumor*

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It is a matter of common knowledge that in human cancers the number of metastases bears no relation to the size of the primary tumor. Since the factors responsible for this lack of correlation are not understood, it seemed of interest to inquire into their identity. However, with human tumors it is difficult—or even impossible—to investigate this problem quantitatively, because in man the variety of neoplasms is great, the environmental conditions are dissimilar, and the hosts are of widely different constitution. To obviate these unmanageable variables, it is highly desirable to use a standardized transplantable tumor in hosts that have been inbred for many generations and that are kept under uniform environmental conditions. The present experiments, using Sarcoma 241 in C57 mice, were designed to meet these requirements. Their purpose was to ascertain whether the number of metastases is related to the size and duration of growth of a primary tumor.

Since the process of metastasis is primarily dependent upon embolism of tumor cells, it was decided to determine first the quantitative relationship between the number of viable cells artificially introduced into the circulation and the number of metastatic tumors so produced. If it could be shown that a strongly positive correlation exists, then variations in the number of "spontaneous" metastases probably could be attributed to factors in the primary tumor that affect the number of emboli released into the circulation.

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service.

[1] The reliability of this method was established by staining a suspension of polymorphonuclear leukocytes which were then examined for motility. Motile cells were unstained and refractile. Nonmotile cells were stained and nonrefractile.

Received for publication, February 5, 1950.

RELATIONSHIP OF THE NUMBER OF EMBOLIC CELLS TO THE NUMBER OF RESULTANT TUMORS

MATERIALS AND METHODS

A suspension of tumor cells was prepared by forcing mouse Sarcoma 241 (1) through the mesh of a stainless steel screen, and by adding a few cc. of a mixture of equal parts of serum and physiological salt solution. This suspension was allowed to stand for 10 minutes to permit settling of clumped cells. The supernatant suspension used in the experiment consisted almost exclusively of single cells.

The number of cells per cubic centimeter of this suspension was determined with a counting chamber. Because many of the cells were damaged by the sieving procedure, it was necessary to ascertain the percentage of living cells. This was done by using trypan blue as an indicator of viability.

A dilute solution of trypan blue was made in a mixture of salt solution and serum. Cells were mixed with this solution and observed in a counting chamber. Under these conditions, viable cells are unstained and refractile, whereas the nuclei of dead cells are stained blue and the cells are not refractile.[1] By this method it was found that only about 6 per cent of the suspended tumor cells in this particular suspension were viable.

Serial dilutions of the original suspension were made, using the saline-serum mixture as the diluent. These suspensions were then injected into the tail veins of C57 mice, using 0.3 cc. for each injection. Eighteen days later the mice were sacrificed, and the tumors in the lungs were counted with the aid of a dissecting microscope.

RESULTS

The results are shown in Table 1. There was direct proportionality between the number of cells injected and the number of resultant pulmonary tumors. It is also apparent that relatively

large numbers of embolic cells were needed to produce a tumor, indicating that most of the cells failed to survive the process of embolism and lodgement in their new site, the lungs.[2]

TABLE 1

RELATIONSHIP OF NUMBER OF VIABLE EMBOLIC TUMOR CELLS TO THE NUMBER OF RESULTANT PULMONARY TUMORS

Number of mice	Number of viable cells injected	Average number of pulmonary tumors
10	104,400	11.5
10	19,800	2.5
8	5,400	1.7
10	900	0.3

RELATIONSHIP OF THE SIZE AND DURATION OF GROWTH OF THE PRIMARY TUMOR TO THE NUMBER OF METASTASES

Having established the fact that the number of metastases is directly proportional to the number of viable embolic cells, the question may now be asked: Do the size of the primary tumor and the duration of its growth determine the number of emboli released?

MATERIAL AND METHODS

The material selected was mouse Sarcoma 241 implanted in C57 mice. In this highly inbred strain, 100 per cent of "takes" occur and "spontaneous" pulmonary metastases appear almost invariably.

Sixty-two mice were inoculated subcutaneously in the flank with fragments of tumor approximately 3 mm. in diameter and of as uniform a size as possible. Mice were sacrificed at intervals, the lungs were removed, and the subpleural metastases were counted with the aid of a dissecting microscope. As a check on these counts, a pulmonary lobe from each of four mice was sectioned serially, and metastases were counted with the microscope. In this way, a few additional subpleural tumors of microscopic size and a still smaller number of more deeply located tumors were picked up, but the counts on serial sections were proportional to counts made by the method described above—which thus appeared satisfactory for the present purpose. The ultimate sizes attained by the primary tumors were determined by measuring the volume of fluid displaced by them when immersed in isotonic salt solution.

RESULTS

Effect of the duration of growth.—The period of growth of the primary tumor on the number of pulmonary metastases is shown in Table 2. There is a consistent increase in the number of secondary tumors with time, beginning on the eleventh day and reaching a peak on the 21st. Thus, duration of growth of the primary tumor was found to be an important factor in affecting the number of metastases.

Effect on the number of metastases of the original size of the tumor implant.—Experiments were designed to answer the question: Is the number of pulmonary metastases proportional to the size of the tumor implant? Twenty mice were inoculated in one flank with large tumor fragments measuring about 5 mm., and twenty mice (of which eighteen survived) with small fragments measuring about 2 mm. All animals were sacrificed on the seventeenth day. When the pulmonary metastases were counted, a very wide range in their numbers was found—from 0 to 105. The mean number of metastases resulting from large fragments was 21.7, with a standard error of ± 5.41. In mice inoculated with small fragments, the mean with its standard error was 9.4 ± 1.89. The ratio of the difference (12.3) to its standard error was 2.158, which corresponds to a probability of between 0.03 and 0.04. In other words, a difference of this (or greater) magnitude would occur by chance only 3 or 4 times in 100, and therefore is regarded as significant. In this experiment, therefore, greater numbers of metastases resulted from implantation of large tumor fragments than from small ones.

Relation of the number of metastases to the final size of the primary tumor.—The data given above were based on the *original* size of the fragments of tissue at the time of implantation. Now, the relationship of the number of metastases to the *final* size of the resulting tumors, as determined at the conclusion of the experiment 17 days later, will be considered.

TABLE 2

THE EFFECT OF DURATION OF GROWTH OF PRIMARY TUMORS ON THE NUMBER OF PULMONARY METASTASES

Duration of growth of primary tumors (days)	Number of animals	Average number of metastases
9	8	0
11	4	4.0
12	10	1.2
14	2	1.0
15	12	3.8
17	4	8.2
19	8	11.2
21	11	25.8
26	3	13.7

The volumes obtained were arranged in numerical order and divided into two groups of nineteen

[2] Such a mortality of embolic cells is consistent with the conclusions of Schmidt (4), Iwasaki (3), and Warren and Gates (5). See also Willis (6).

each, one group containing the larger values (ranging from 4.5 to 1.8 cc.), the other the smaller values (ranging from 1.75 to 0.30 cc.). Comparison was then made between the number of metastases corresponding to each group. In those animals having the larger primary tumors, the mean number of metastases was 19.8, with a standard error of ± 5.74; the group with smaller tumors had 11.8 ± 2.25 metastases. However, because of the wide scatter in numbers of metastases, the difference between the two groups cannot be regarded as significant ($P = .19$). Likewise, when final volumes of primary tumors are correlated with numbers of metastases, the value of the correlation coefficient is 0.18, and $P = >0.1$, a value considerably short of significance. It is, therefore, concluded that in this experiment the number of pulmonary metastases was not definitely related to the final size attained by the primary tumor.

DISCUSSION

As demonstrated in these experiments, the likelihood of metastasis from a cancer depends on the number of emboli released from the primary tumor. It depends also on the ability of emboli to establish themselves and grow where they lodge, as discussed by others (3–6) and in recent (2) and forthcoming papers from this laboratory. In the present investigation, attention centers on factors in the primary tumor regulating the number of emboli given off. Among the most important of such factors may be reckoned the ability of cancer cells to invade vessels and there to release emboli into the circulation. If these properties can be held relatively constant by using highly standardized transplantable tumors in inbred hosts, such simple physical factors as the size of the primary tumor and the duration of its growth would be expected to affect the number of metastases to a demonstrable extent. It is, therefore, surprising to find that even under these standardized conditions there is little correlation between the size of the primary tumor and the number of metastases it produces. It may be assumed, therefore, that the wide variation in numbers of "spontaneous" metastases must depend on factors existing in the primary tumor, as yet unidentified, that affect the number of emboli given off.

SUMMARY AND CONCLUSIONS

Experiments were designed to determine the relationship between the number of embolic viable tumor cells and the number of resultant tumors. Serial dilutions of a suspension of viable cells from mouse Sarcoma 241 were injected into the tail veins of C57 mice. A direct proportionality was found between the number of cells injected and the number of tumors resulting in the lungs. It was also shown that very few tumors were formed in relation to the number of tumor cells injected, indicating a high mortality of the cells.

Further experiments were designed to determine the relationship of size and duration of growth of the primary tumor to the number of emboli released into the circulation. Using C57 mice with transplantable Sarcoma 241 it was found that:

1. The longer a primary tumor existed, the greater the number of emboli released, as judged by the number of metastases appearing in the lungs.
2. Mice inoculated with large tumor fragments had a greater number of metastatic tumors than did mice inoculated with smaller fragments.
3. There was not a significant correlation between the number of pulmonary metastases and the final size attained by the primary tumors.

It is concluded from these experiments:

1. That the number of metastases is directly proportional to the number of viable tumor cell emboli released into the circulation.
2. That although the number of emboli released may be affected by such simple factors as the initial size of the tumor inoculum and duration of growth of the primary tumors, it depends to an even greater extent upon factors within the primary tumor as yet unrecognized.

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The Effect of Methylcholanthrene on the Genital Tracts of Rabbits and Dogs as Revealed by Biopsies and Exfoliative Cytology*†

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This is a study of the effect of methylcholanthrene applied to the genital tracts of female rabbits and dogs. An attempt was made to produce tumors of the cervix and vagina in order to follow their natural history by serial studies of the exfoliated cells and biopsies. Unfortunately, the genital tracts of these animals proved resistant to the carcinogen, and tumors did not result.

TABLE 1

Summary of Results

Animal	Preparation	Site of Application	Survival	Remarks
4 Rabbits	1 per cent methylcholanthrene in benzene, once weekly	4 cm. above vaginal orifice inside vagina	20, 30, 46, 76 wks.	No tumors
5 Rabbits	30 mg. methylcholanthrene in 0.2 cc. paraffin	Injected at squamo-columnar junction of vagina	68, 68, 68, 73, 73 wks.	No tumors
4 Dogs	100 mg. methylcholanthrene in 1 cc. paraffin	Injected into cervix uteri	47, 47, 59, 67 wks.	No tumors
3 Dogs	1 per cent methylcholanthrene in benzene	Painted on cervix once weekly	164, 183, 190 wks.	Leukoplakia in one, no changes in two

METHODS

Methylcholanthrene was selected as the carcinogen, because it has produced tumors at other sites in these animals (2, 5).

Originally, an attempt was made to visualize the cervices of rabbits in order to paint them with methylcholanthrene in benzene. However, because of technical difficulties, the cervix was soon abandoned as the site of application of the carcinogen, and an area on the vaginal wall, 4 cm. above the vaginal orifice, was selected. Glass tubing was inserted into the vagina for a distance of 4 cm. and 1 per cent methylcholanthrene in benzene was applied once weekly to the vaginal walls of four rabbits by means of cotton applicators. Benzene was selected as the solvent because Murphy and Sturm (3) found it to be a "neutral" solvent which neither promoted nor retarded carcinogenesis.

In another group of rabbits, 30 mg. of methylcholanthrene in a vehicle of 0.2 cc. of special low melting point (43° C.) paraffin was injected into the submucosa of the vagina at the squamo-columnar junction which is near the vaginal orifice. The frequency of carcinogenesis at this junction in the human cervix, as well as the occurrence of spontaneous carcinomas in rabbits at this site, as reported by Greene and Newton (1), suggested this area as the most receptive place for the injection of methylcholanthrene. For control purposes, two rabbits were injected in the same area with paraffin only.

Laparotomies were performed on six dogs to expose the cervices, and 100 mg. of methylcholanthrene in 1 cc. of special low melting point (43° C.) paraffin was injected into the cervix beneath the squamo-columnar junction. Cervices of two dogs were injected with paraffin only.

* Abridgement of thesis submitted by W. C. Pallas to the faculty of the Graduate School of the University of Colorado in partial fulfilment of the requirements for the degree of Master of Science in Obstetrics and Gynecology.

† This project has been supported by grants from the U.S. Public Health Service and the Committee on Research and Creative Activities, University of Colorado.

Received for publication, February 9, 1950.

Bi-weekly vaginal smears were taken from all the above animals and were stained and examined by the Papanicolaou (4) method.

In four more dogs the cervices were exteriorized, and weekly applications of 1 per cent methylcholanthrene in benzene were made. Controls consisted of one dog with an exteriorized cervix otherwise untreated, and another dog to which only benzene was applied. These animals were observed by serial biopsies every 3 months.

No neoplastic cells were observed in the vaginal smears. In occasional specimens there were numerous polymorphonuclear leukocytes and epithelial cells with enlarged nuclei, a few other atypical cells, and an increased number of stratified squamous cells. Autopsy findings failed to reveal any malignant tumors.

SUMMARY AND CONCLUSIONS

An unsuccessful attempt was made to induce malignant tumors by methylcholanthrene in the cervices and vaginas of rabbits and dogs. The vaginas of rabbits were exposed to the carcinogen for as long as 76 weeks. The uterine cervices of the dogs were exposed to the same substance for as long as 190 weeks. Apparently, the genital tracts of these animals are very resistant to methylcholanthrene. Since no tumors were produced, it was impossible to study the histogenetic evolution of artificially produced tumors.

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Studies on the Hazard Involved in the Use of C^{14}

II. The Effect of a Single Dose of C^{14}-labeled Sodium Bicarbonate on the Pattern of Deaths from Spontaneous Leukemia in Akm Mice*

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As a portion of a study designed to obtain data which might be useful in assessing the hazard involved in research with carbon 14, it seemed important to determine the effect of this isotope in a chemical state ($C^{14}O_2$), apt to be encountered in the laboratory, on a type of neoplastic disease (leukemia) known to be influenced by radiation. Choice of the dosage level employed in the experiment was dictated by a consideration of the quantities of carbon 14 used in ordinary scale tracer experiments. The selection of a strain of mice which develop and die of spontaneous leukemia in almost 100 per cent of cases was based on the desire to obtain as much quantitative data as possible in a reasonable time, with the greatest economy of isotope.

TABLE 1

THE PATTERN OF DEATHS FROM SPONTANEOUS LEUKEMIA

Akm mice given a single dose of $NaHC^{14}O_3$ (18 μc.) compared with litter-mate controls injected with nonradioactive $NaHCO_3$

CONTROLS					$NaHC^{14}O_3$ INJECTED				
Animal no.	Sex	Litter	Age when injected* (days)	Age at leukemic death (days)	Animal no.	Sex	Litter	Age when injected† (days)	Age at leukemic death (days)
C56	F	a	97	286	RA56	F	a	97	282
C57	F	a	97	244	RA57	F	a	97	216
C58	F	a	97	300	RA58	F	a	97	274
C59	F	b	84	252	RA59	F	b	84	259
C60	F	b	84	294	RA60	F	b	84	273
C61	M	c	95	NLD‡	RA61	M	c	95	241
C62	M	d	92	323	Ra 62	M	c	95	252
C63	M	d	92	NLD‡	RA63	M	d	92	397
C64	M	d	92	319	RA64	M	d	92	249
C65	M	e	92	363	RA65	M	d	92	252
C66	M	f	103	372	RA66	M	f	103	260
C67	M	g	98	386	RA67	M	g	98	359
C68	M	g	98	203	RA68	M	g	98	479
C69	M	h	98	295	RA69	M	h	98	408
C70	M	i	98	341	RA70	M	i	98	423
Average:			94.5	306				95	308

* Nonradioactive sodium bicarbonate.

† $NaHC^{14}O_3$ (18 μc.).

‡ Nonleukemic deaths 2 days after injection.

Note: No osteogenic tumors were observed in any of the above animals.

Previous studies of the over-all body radiation of mice following injection of 18 microcuries (μc.) of $NaHC^{14}O_3$ (man-equivalent 50 millicuries) indicated an integrated exposure of about 0.11 roentgen-equivalent-physical (r.e.p.) for the first 24 hours and an average daily dose of about 0.003 r.e.p. for the period of 1–3 months (1, 2).

* This work was supported by a grant from the Atomic Energy Commission, administered by the Office of Naval Research.

Received for publication, February 10, 1950.

EXPERIMENTAL

The mice employed in this study were of the Akm strain, brother-sister inbred for genetic control.

It was previously determined that the average life span of 107 untreated Akm strain mice raised in this laboratory was 280.2 days (± S.D. 51.3 for individual animals or ± S.D. 14.2 for seven random groups of fifteen mice each); and the effect of a given dose of carbon 14-labeled sodium bicarbonate on the pattern of deaths from the spontaneous leukemia which develops in this strain was studied as follows:

TABLE 2

SUMMARY OF DATA ON EFFECT OF INJECTION OF $NaHC^{14}O_3$ ON PATTERN OF SPONTANEOUS LEUKEMIA IN AKM MICE

	Controls	$NaHC^{14}O_3$
Mortality data	13/13	15/15
Average age at leukemic death (days)	306	308
Standard deviation	±51.2	±79.0

Note: The average life span of 107 untreated Akm strain mice dying of spontaneous leukemia in this laboratory during the past year was 280.2 days with a standard deviation of ± 51.3.

Paired Akm strain litter mates of the same sex and approximately the same weight were divided into two groups of fifteen mice each. The ages of the individual mice are indicated in Table 1. The controls were injected I.P. with 100 mg/kg of nonradioactive sodium bicarbonate, while the experimental group was injected with a similar level of radioactive $NaHC^{14}O_3$ (18 μc/mouse). The experimental group was then placed in a large metabolism chamber, where it was maintained for a period of 1 week. After this 1-week period, considered necessary for collection of exhaled $C^{14}O_2$ from the experimental group, both groups were set aside in an air-conditioned animal room to await onset of and death from spontaneous leukemia. Both groups of animals were fed laboratory chow supplemented by bread and milk and raw carrots.

RESULTS AND DISCUSSION

The pattern of deaths from leukemia observed in the two groups is shown in Table 1. A summary of the data obtained is presented in Table 2.

The amount of carbon 14 injected into mice of the experimental group in the present study was equivalent to a dose of about 50 mc. for a 70-kg. man on a weight basis. Such a dose, it would seem, is not likely to be inadvertently taken into the body of a cautious experimenter in an ordinary investigation. However, this level is not an unreasonable requirement for certain types of human experimentation if and when such use of carbon 14 is allowed by the Atomic Energy Commission.

It seems quite clear from the data that under the conditions of this experiment a single dose of 18 μc. of carbon 14 as $NaHC^{14}O_3$ has had no significant effect on the pattern of deaths from leukemia in this highly susceptible strain of mice.

SUMMARY

Leukemia-susceptible Akm mice have been injected with carbon 14-labeled sodium bicarbonate at the rate of 18 μc. per mouse. This dose has had no significant effect on the pattern of deaths from leukemia in this strain of animals.

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Neoplasms in Rats Treated with Pituitary Growth Hormone

II. Adrenal Glands*

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The occurrence of many different neoplasms in fifteen normal plateaued female rats treated for 350 to 485 days with pituitary growth hormone[1] was reported in the preceding paper of this series (4). The histogenesis of pulmonary lymphosarcoma was reported in some detail in that paper. The present report is concerned with the hyperplastic and neoplastic changes in the adrenal glands of the same animals.

The adrenal glands of the rats injected with pituitary growth hormone and their controls were dissected free of fat, weighed, and fixed. They were usually embedded in paraffin, sectioned serially, and stained with hematoxylin and eosin, except in one of the control rats and in three of the experimental rats—in which instance frozen sections were prepared and stained with Sudan Orange and hematoxylin.

RESULTS

Control Rats Injected with Albumin

The adrenal glands of the control rats were fairly uniform in appearance, and the weight of a single gland varied from 23 to 48 mg. (Table 1 and Figs. 1–3). These glands presented the usual lenticular shape and were pale yellow. Microscopically, the glands were characterized by cortices of uniform thickness. The cortical tissue was differentiated into the usual three zones. The zona glomerulosa was narrow; the cells were small, with scanty cytoplasm, and were arranged into small groups.

* Aided by grants from the U.S. Public Health Service RG-409; the Research Board of the University of California; the American Cancer Society, Inc., N.Y.; and the University of California Cancer Grant.

[1] The pituitary growth hormone used in this study was pure by physico-chemical criteria (2). The initial daily dose of 0.4 mg. was gradually increased to a maximum of 3.0 mg. daily by the end of the experiment.

Received for publication, February 15, 1950.

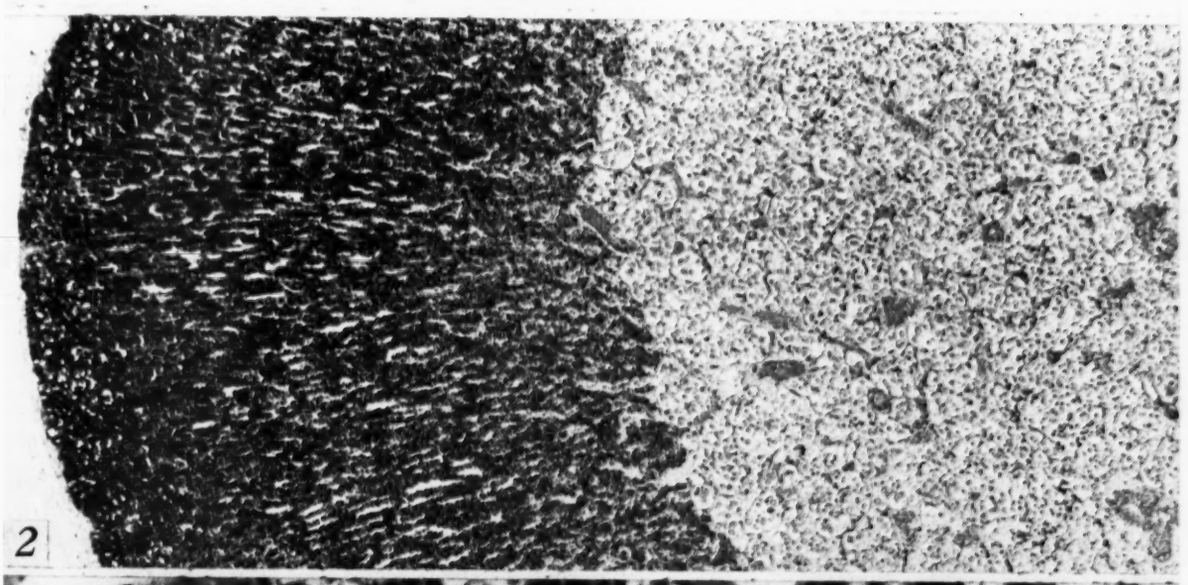
The zona fasciculata was composed of long, straight cords of cells. The cells in this zone were larger than those of the zona glomerulosa, and the cytoplasm of these cells was vacuolated in paraffin sections and filled with lipoid in frozen sections.

TABLE 1

ADRENAL GLANDS OF CONTROLS

Rat	Right or left	Weight (mg.)	Abnormalities noted: Cortex	Medulla
B6530	R	53		
	L			
B6512	R	48		
	L		nodular	
B6400	R	28		
	L	37		
B6508	R	38		
	L	47		
W6523	R	30		
	L	32		
GH6442	R	30		
	L	30		
GH6554	R	30		
	L	30	small nests of medullary cells in cortex	
GH6357	R	30		
	L	38		
G6460	R	54	nodular	
	L		nodular	
GH6262	R	48		
	L	40		
G6268	R	40	nodular	
	L	36	nodular	
B6291	R	36		
	L	34		
BH6292	R	36		
	L	38		
B6492	R	24		
	L	23		
BH6294	R	32		
	L	44		

The reticular zone consisted of cells arranged in irregular cords; these cells were slightly smaller than those of the zona fasciculata. The cytoplasm of these cells contained small amounts of brown pigment. The nuclei of the cells of all three zones were small, round, and deeply basophilic. In three



FIGS. 1–3.—Control Rats; adrenal glands.

FIG. 1.—Rat B 6530. Topographic view; frozen section; hematoxylin and Sudan Orange × 16. Note the normal perivascular extension of medulla to hilus.

FIG. 2.—Higher magnification of Fig. 1, × 62.

FIG. 3.—Rat B 6400. Paraffin section; hematoxylin and eosin. The cortical cells have finely granular, dense cytoplasm and small, round, deeply-staining nuclei. The medullary cells have pale or clear cytoplasm and usually vesicular, round nuclei. A few spindle cells with hyperchromatic nuclei are seen. × 355.

rats, the zona fasciculata of both adrenal glands was nodular. The nodular areas were composed of cells which had more abundant cytoplasm than those of the adjacent cortex. These areas had a "barrel stave" appearance due to the arrangement of the cells into slightly curved cords.

The medullae of the glands of nearly all animals were uniform in shape and in amount. The medullary tissue was composed of polyhedral, flat or crescentic cells usually arranged in small compact groups. The predominant cell was the large polyhedral cell with a round vesicular nucleus and pale or clear cytoplasm. The flat crescentic cells had hyperchromatic nuclei and scanty granular cytoplasm. In one rat (GH 6554), several small nests of medullary cells extended into the zona reticularis. No neoplasms of the adrenal glands were present in the control rats.

Experimental Rats Injected with Pituitary Growth Hormone

The weights of the adrenals of the rats injected with growth hormone ranged from 27 to 220 mg. (Table 2). Small nodules of translucent pink and hemorrhagic medullary tissue presented on the surface of one of the adrenals in each of three rats (GH 6368, BH 6334, and BH 6276). In rats BH 6334 and BH 6276 these nodules were not related to the vascular pole of the adrenal gland. There was no consistent increase in the width of the adrenal cortices of rats injected with growth hormone; in some animals, the cortices were narrower than those of the controls. Nodular areas were present in the zona fasciculata of six of the rats. These areas were similar to those observed in the adrenal cortices of two of the control rats. In one rat there was a cortical adenoma in the zona reticularis (Figs. 6 and 9). In many of the rats there was displacement and infiltration of the cortex by the medullary proliferation described below.

The medullae of all adrenals of the experimental rats were enlarged; this was considered the basis for the increased weight of the adrenals in many of these rats. The medullary cells had more abundant, pale cytoplasm and larger, vesicular nuclei. Nucleoli were often prominent. Rare mitoses were present. Most of the cells were polyhedral, but some were spindle-shaped. Except for their larger size, the medullary cells were essentially similar to those of the control rats.

In addition to the medullary hypertrophy and hyperplasia, there were focal or nodular areas of varying size composed of atypical cells in the medullae of nine rats (Figs. 4 and 5). These areas were present in one adrenal in each of six rats and in both adrenals in three rats. The cells in most of these areas were small, with scanty cytoplasm and hyperchromatic nuclei. Occasional mitoses were present. Frequently, the cells had indistinct borders and were in masses suggesting a syncytium.

In some of the areas of atypical medullary hyperplasia, the cells were larger than those of the adjacent medulla. These cells had more abundant cytoplasm. The nuclei were larger; the nuclear membranes were thicker, but the vesicular appearance of the nuclei was similar to that of the adjacent medulla. There were occasional mitoses.

There were transitional stages between all types of cells described above.

In many of these rats, the zona reticularis was displaced and invaded by nodules of atypical medullary cells. Groups of medullary cells occasionally infiltrated as far as the zona fasciculata.

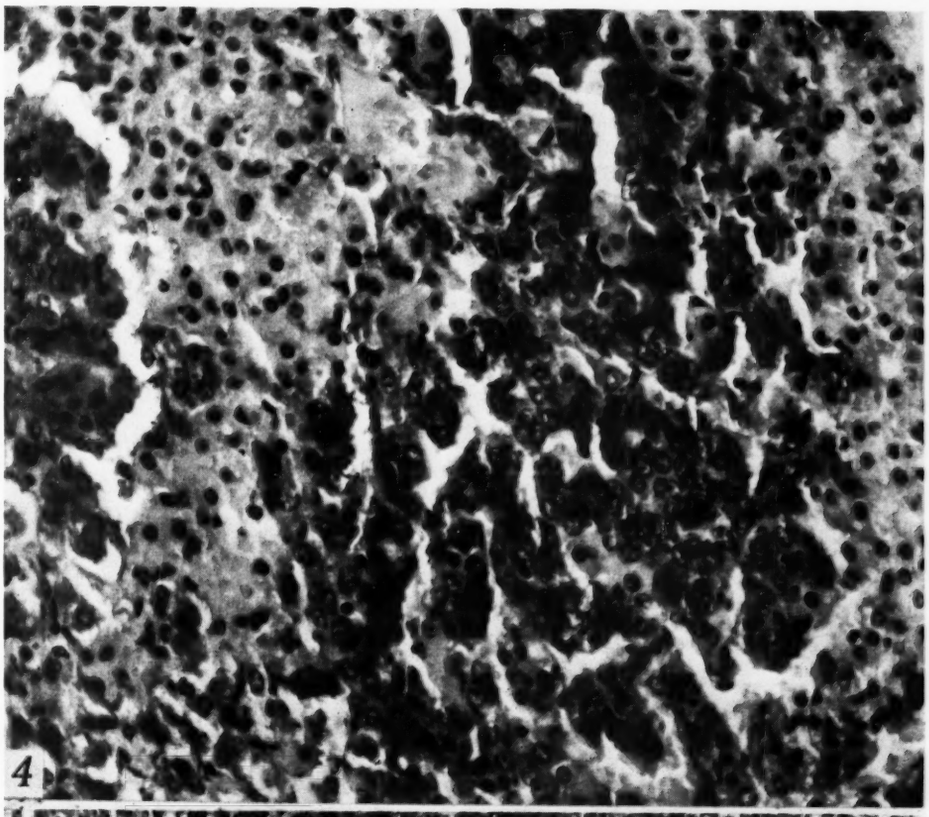
In two rats (BH 6334 and BH 6276) the atypical

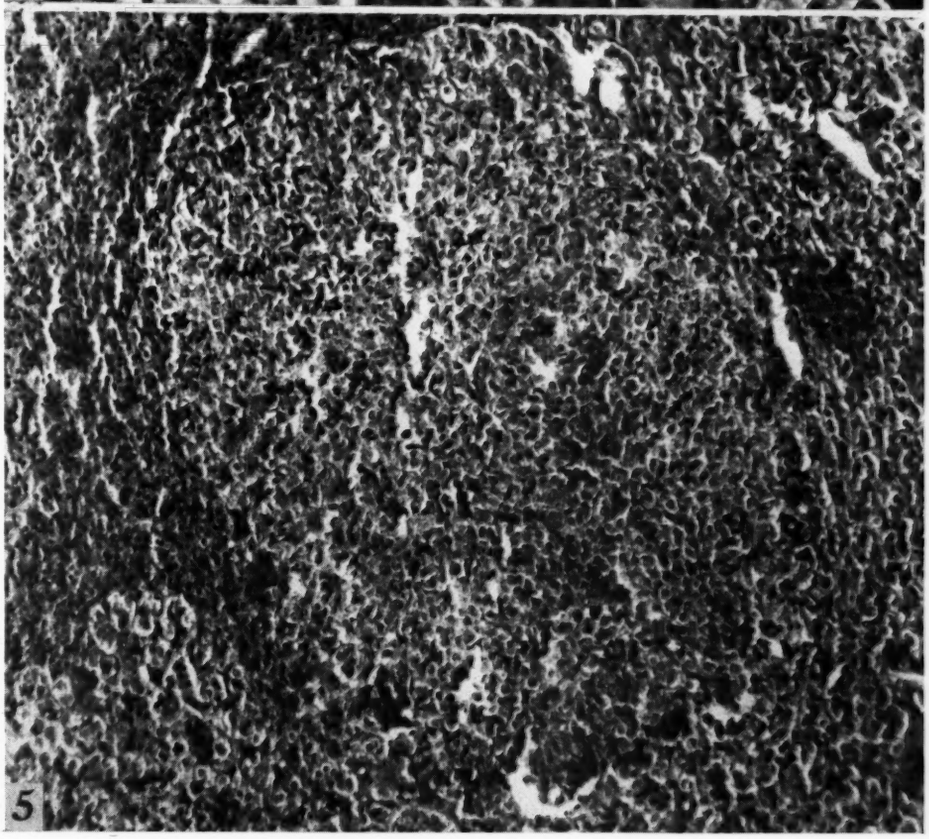
TABLE 2

Adrenal Glands of Experimental Rats

Rat	Right or left	Weight (mg.)	Abnormalities noted: Cortex	Medulla
G6534	R	121		hyperplasia
	L			hyperplasia
B6458	R	100		hyperplasia
	L			
GH6368	R	49		focal atypical hyperplasia
	L	220	displaced	hyaline degeneration of large medullary tumor
BH6274*	R	59	displaced	bilateral metastatic lymphosarcoma and atypical hyperplasia
	L	88	displaced	
BH6334	R	113	nodular and displaced	large medullary tumor
	L	72		focal atypical hyperplasia
G6506	R	95		hyperplasia
	L	92		focal atypical hyperplasia
BH6276	R	40	adenoma	hyperplasia
	L	40		medullary tumor growing through capsule
G6553	R	27		hyperplasia
	L	31		hyperplasia
BH6313	R	61		hyperplasia
	L	71		focal atypical hyperplasia
W6324	R	66		focal atypical hyperplasia
	L	73	nodular	focal atypical hyperplasia
G6255	R	40	nodular	hyperplasia
	L	38	nodular	hyperplasia
G6361	R	40	nodular	hyperplasia
	L	32		focal atypical hyperplasia
B6266	R	36		focal atypical hyperplasia
	L	50	nodular	hyperplasia
G6269	R	44		hyperplasia
	L	50		focal atypical hyperplasia
G6271	R	40		focal atypical hyperplasia
	L	48		hyperplasia

* Rat BH6274, as previously reported, had lymphosarcoma involving both lungs and hilar lymph nodes, with invasion of the right pulmonary vein and left atrium.

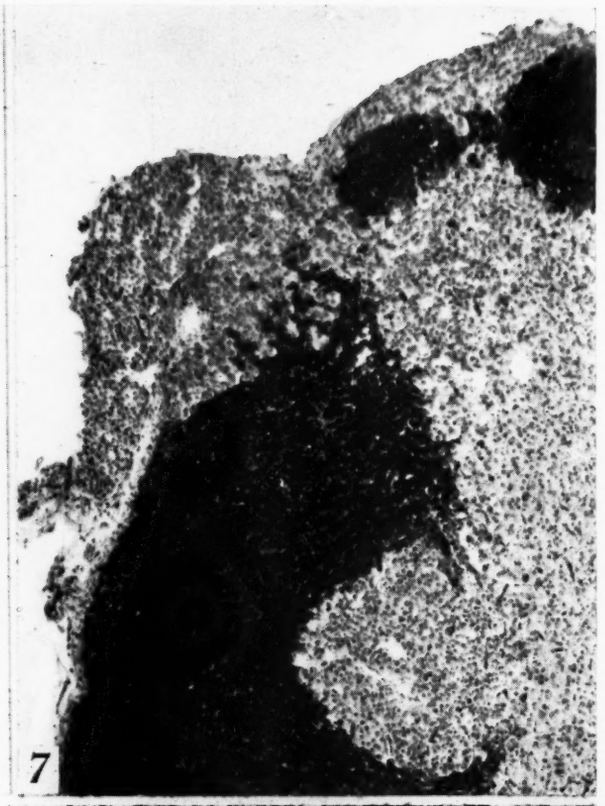




FIGS. 4–5.—Experimental Rats; adrenal glands.

FIG. 4.—Rat G 6271. There is displacement and invasion of the zona reticularis of the cortex by small "dark" atypical medullary cells in the center. These cells have indistinct borders, hyperchromatic nuclei, and syncytial masses. There is another nodule of atypical medullary cells at the extreme left. There is some compression and distortion of the zona reticularis between these two nodules. There are a few typical hypertrophic medullary cells at the lower left. × 355.

FIG. 5.—Rat B 6266. There are nodular masses of large atypical medullary cells invading and compressing cortex. × 125.



FIGS. 6–9.—Experimental Rat BH 6276; adrenal gland; frozen section; hematoxylin and Sudan Orange.

FIG. 6.—Topographic view; note the cortical adenoma and the irregular outlines of the medulla. × 16.

FIG. 7.—Invasion of cortex by neoplastic medullary cells. The proliferating medullary cells at the upper left have grown through the cortex and have formed a nodule on the surface of the adrenal gland. × 62.

FIG. 8.—Higher magnification showing disruption of cortex and capsule by neoplastic medullary cells. × 125.

FIG. 9.—Higher magnification of cortical adenoma. × 125.

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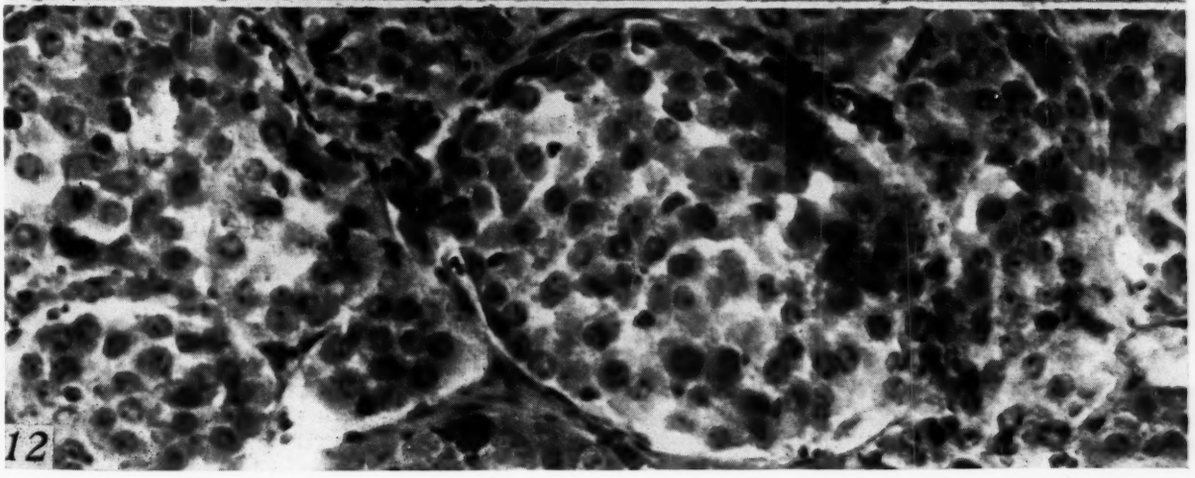

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FIGS. 10–12.—Experimental Rat BH 6334; adrenal gland; frozen section; hematoxylin and Sudan Orange.

FIG. 10.—Topographic view; the adrenal is greatly enlarged (compare with Fig. 1). The distribution of cortical lipoid is altered due to the nodularity of the cortex. Neoplastic medullary cells have broken through the cortex and attenuated the capsule at the right. There is a large nodular area of abnormal medullary cells to the left. × 16.

FIG. 11.—Higher magnification of the nodular area of neoplastic medullary tissue which is displacing the cortex. The pale area in the center of the picture is due to degeneration and old hemorrhage. × 62.

FIG. 12.—Higher magnification of neoplastic medullary cells. The nuclei vary in size and are frequently hyperchromatic; the cytoplasm is finely granular. The cells are arranged in compact groups. × 355.

medullary hyperplasia with cortical displacement and infiltration was of such proportions that the proliferating medullary tissue could be observed on the surface of the adrenal gland as a macroscopically visible tumor (Figs. 6–12). In rat BH 6276, the medullary cells had broken through the capsule (Figs. 7 and 8). The medullary cells were large and were arranged in small, compact groups. The cells were polyhedral or spindle-shaped. The nuclei of the polyhedral cells were moderately vesicular and varied considerably in size; those of the spindle-shaped cells were hyperchromatic. The nuclear membranes were thick. There were occasional mitoses. In one rat (G 6368), most of the medullary tissues had undergone hyaline degeneration. In one area, the outlines of the pre-existing medullary cells were faintly discernible. A few macrophages filled with hemosiderin were present in the center and about the periphery of the hyaline mass. There was a slender rim of large, atypical medullary cells along one edge of the hyaline nodule. The lesion in this rat was interpreted as a medullary tumor which had undergone hemorrhage, infarction, and hyaline degeneration.

Metastatic adrenal medullary tumors were not observed in any of the rats.

There was no significant difference between the ratio of heart weight to body weight in the rats injected with growth hormone, as compared with the control rats injected with albumin. Nor was there any evidence of arteriolosclerosis in any of the animals.

DISCUSSION

The adrenal cortices were nodular in six of fifteen experimental rats; one of the other experimental rats had an adrenal cortical adenoma. Only two of the control rats had nodular adrenal cortices. The significance of this observation is not clear, particularly in view of the fact that the adrenal cortices of the experimental rats showed no other evidence of stimulation.

Hypertrophy of the adrenal medullae of rats treated repeatedly with thiouracil has been reported by Marine and Baumann (3). These authors suggest that a direct interrelationship between the thyroid gland and the adrenal medulla was responsible for the medullary hypertrophy that they observed. However, their studies did not exclude the possibility of such hypertrophy being mediated through the pituitary gland.

It was reported previously that the adrenal medullae of rats repeatedly injected with growth hormone were larger than those of control rats. This was true not only for normal plateaued female rats (1) receiving growth hormone but also true for hypophysectomized female rats (5) receiving growth hormone. The occurrence of a medullary tumor in a previous group of normal plateaued female rats treated similarly was also reported (1). In the present group of rats, as well as in those previously reported, the thyroid glands of the rats injected with growth hormone were morphologically indistinguishable from that observed in the controls.

The remarkable frequency with which proliferative (hyperplastic and neoplastic) lesions of the adrenal medulla occurred in the present group of rats injected repeatedly with growth hormone, and the absence of similar changes in the control rats, indicates that these changes are directly related to the prolonged administration of preparations of growth hormone. The displacement and invasion of the cortex by groups of atypical medullary cells was very conspicuous. There were almost all stages of development between the focal areas of atypical medullary cells and the medullary tumors which had grown through both cortex and capsule of the adrenals. For this reason, the focal areas of atypical medullary cells are in all probability early neoplastic lesions.

SUMMARY AND CONCLUSIONS

1. The adrenal cortices of rats injected chronically with pituitary growth hormone showed an increase in nodularity as compared with the controls. There was no other evidence of stimulation of the adrenal cortices of the experimental rats.

2. The adrenal medullary tissue was increased in amount in all rats injected chronically with growth hormone. The increase was due to cellular hypertrophy and proliferation.

3. In ten of fifteen rats injected chronically with pituitary growth hormone, there were areas of neoplastic cells displacing and invading the adrenal cortex. In three of the rats, the neoplastic medullary tissue had grown through the cortex and was macroscopically visible on the surface of the adrenal gland.

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Plasma Antitrypsin Levels during Growth of a Rat Fibrosarcoma

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The rise of antitrypsin concentrations in patients with malignant neoplasms has been reported by us (5) and by several others (1–3). However, no evaluation of the levels at the time of origin or during the growth of the tumor was possible. It seemed desirable, therefore, to ascertain whether antitrypsin values would be useful in determining the stage of tumor development and in what circumstances these values could be misleading. Since it was impossible to obtain human material for this *in vivo* study, rats were selected as suitable animals for comparison.

EXPERIMENTAL

Fifty-one male rats of the Sprague-Dawley strain were bled by cardiac puncture and then implanted in the inguinal region with a fibrosarcoma explant. The tumor was originally induced at this hospital with methylcholanthrene and carried through six generations of transplantations. The rats used were fed *ad libitum* on laboratory chow throughout the study. Together with six control rats which received no implants, the implanted rats were bled 10 days after implantation and at 2-week intervals thereafter for 3 months. One cc. of blood was withdrawn with a syringe containing 2 mg. lithium oxalate. The plasma removed from this blood was used in the determination of antitrypsin according to Wells *et al.* (6). At each bleeding the inguinal region was palpated, the size of the implant was noted, and the rat was weighed. White blood counts and differential white counts were made.

RESULTS

On the basis of the tumor activity in the individual rat, the data on the animals were arranged in three groups: I. implant did not grow—nontakes; II. implant grew and then disappeared—regressions; III. implant grew into a well-developed tumor—takes. Group III was subdivided into two sections: (*a*) tumor remained firm and well defined throughout the period of study and (*b*) tumor showed early degeneration and erosion.

Received for publication, February 18, 1950.

Of the 51 animals investigated, 20 fell into Group I, 13 into Group II, 10 into III, *a*, and 8 into III, *b*. After data on each rat had been assigned to a certain group, the antitrypsin levels and blood pictures were studied statistically. In Figs. 1–4, the trends of antitrypsin content and total white counts and lymphocytes are shown for the various groups. The curves were drawn from mean values in scatter diagrams. Of course, greater changes

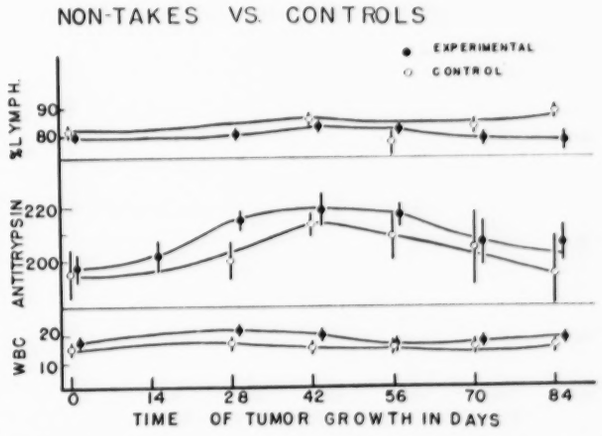

Fig. 1.—Antitrypsin values, total white blood counts, and lymphocyte counts of Group I and controls. White count × 1,000. Vertical lines represent standard deviation from the mean.

could be noted with individual animals, but since most previous reports on humans were given as averages, this method of presentation was selected. As can be seen from the curves, the nontakes showed essentially the same picture as the control animals and leveled off at the same period. In Group II a significant increase in antitrypsin values became apparent at 28 days and gradually returned to normal preimplantation levels. Some interesting observations were made concerning Group III, *b*. Although erosion of the tumor occurred at different stages in individual animals, the respective antitrypsin values consistently decreased to preimplantation levels as soon as necrosis was complete. In three cases the entire tumor was removed at onset of erosion, and the anti-

trypsin concentrations had decreased toward control levels by the time of the next bleeding.

In the four figures the relationship between total white blood count and antitrypsin in tumor

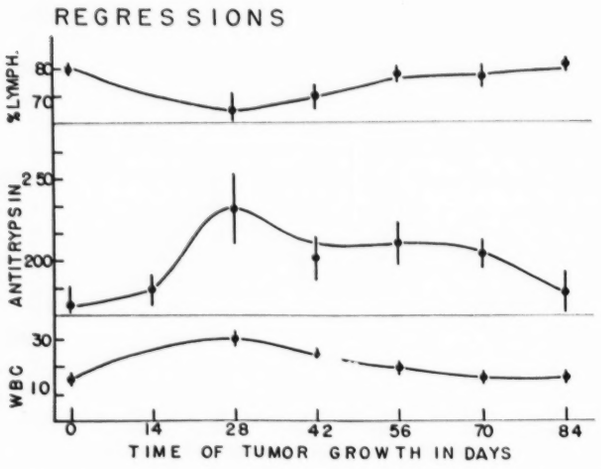

FIG. 2.—Antitrypsin values, total white blood counts, and lymphocyte counts of Group II. White count × 1,000. Vertical lines represent standard deviation from the mean.

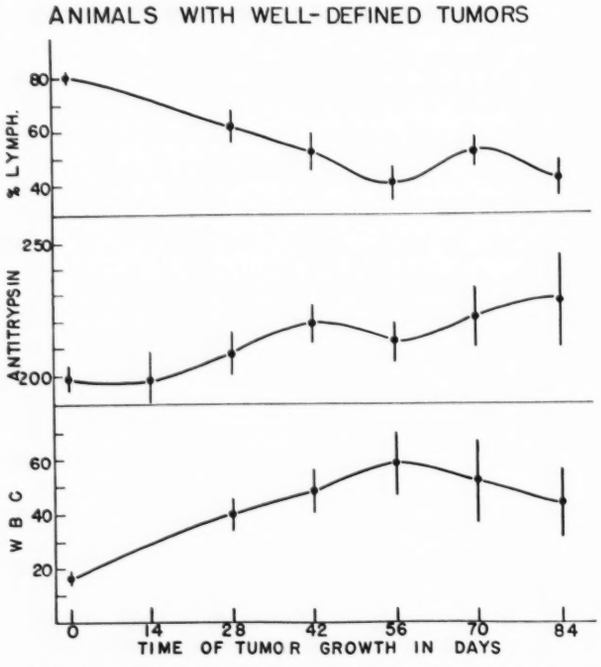

FIG. 3.—Antitrypsin values, total white blood counts, and lymphocyte counts of Group III, *a*. White count × 1,000. Vertical lines represent standard deviation from the mean.

growth is shown. It can be seen from the curves that the two exhibit corresponding variations in all groups. It is interesting to note that the percentages of lymphocytes which are shown in Figures 3 and 4 are inversely proportional to the other two entities. In III, *b* (not shown) the total white counts, like the antitrypsin values, decreased to preimplantation levels after necrosis and after surgical removal of the tumor.

In several of the animals of Group II, a large, fluid-filled cyst formed at the site of implantation. The serosanguinous fluid was removed by aspiration from the sac and its antitrypsin content measured. The amount of antitrypsin it contained was

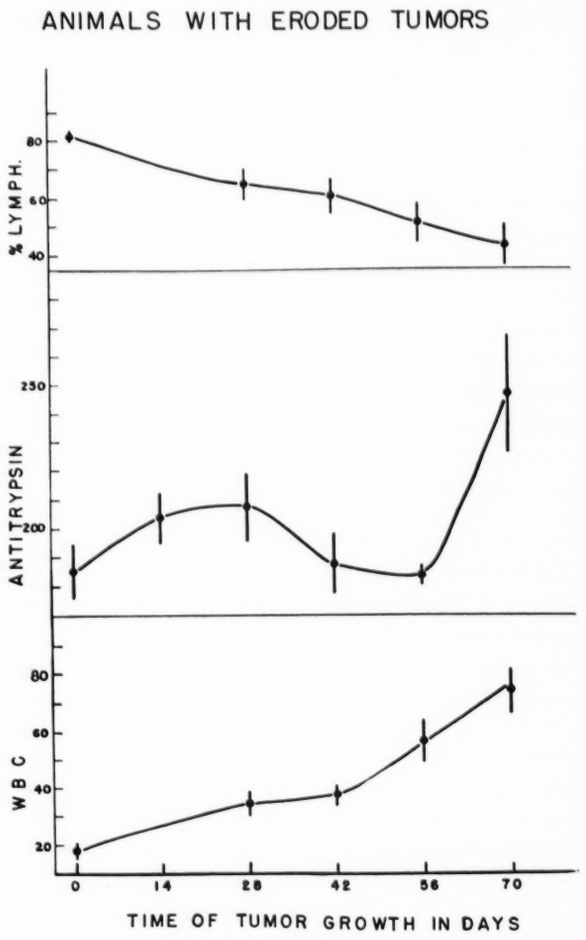

FIG. 4.—Antitrypsin values, total white blood counts, and lymphocyte counts of Group III, *b*. White count × 1,000. Vertical lines represent standard deviation from the mean.

approximately the same as that of normal human plasma. No white cells were demonstrable.

DISCUSSION

In order to deduce any clinical significance from this study, one must remember that rats have a normal antitrypsin and a normal white count which is twice that of humans (4). The fibrosarcoma used is not comparable to any human neoplasm studied. These results, however, lend support to the findings of previous investigations on human cancer patients at this hospital (5). It can be seen that in cases of rapidly growing tumors, the antitrypsin levels and white blood counts are

elevated. Interesting deductions can be made concerning regressing tumors. In this group, as in that in which the tumor continued to grow, the antitrypsin gradually increased to significant values between 28 and 42 days. It is probable, therefore, that this is the "critical period" in which the fate of the tumor is determined. The tumor then either grows or regresses with corresponding elevation or depression of antitrypsin and white blood count. The fact that concentration of antitrypsin and total white blood count so closely parallel each other is indicative of the role played by white cells as carriers of antitrypsin. Since the lymphocyte content varies inversely with the amount of antitrypsin, it would seem that the polymorphonuclear white cells are selectively involved in antitrypsin activity. This is borne out by the fact that neutrophilia appears in the same clinical conditions in which antitrypsin content increases (9). Further work on this subject is needed for proof, but several authors (7, 8) have intimated the role of white cells in tryptic and antitryptic activity. The results found here are most important in corroborating our previous findings—that the determination of antitrypsin may not serve as a diagnosis, but, together with additional clinical data, may give valuable information about the status of the tumor and the health of the patient. These results show that ground work for other problems of this type can be facilitated by studies on rat tumor growth *in vivo*.

SUMMARY

1. Antitrypsin levels and white blood counts at various stages in the growth of a rat fibrosarcoma are reported. There is an elevation of both during the period of most rapid growth.

2. Upon regression and after surgical removal of the tumor, the antitrypsin and white blood count decrease.

3. Control animals and those in which the implant does not take give essentially the same results, with no significant increase in either antitrypsin or white blood count.

4. Serosanguinous fluid accumulating in cysts in regressing tumors contains some antitrypsin but no white cells.

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Potential Sources of Tumor Nitrogen*

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The nitrogen content of a large Walker carcinoma 256 exceeds the amount of nitrogen stored by its host during the period of tumor growth (14). Therefore, part of the nitrogen contained in a tumor must have been derived from the rat's body. Some tissues should then show a decrease in nitrogen content that may be detected by comparing the nitrogen content of the same tissues from tumor-bearing rats and their pair-fed controls of the same age, weight, and sex. The potential sources of tumor nitrogen have been determined in this manner. Apparently, most of the tissues that lose nitrogen during caloric starvation may also lose nitrogen during tumor growth. The liver and spleen, however, acquire nitrogen at least temporarily, while the nitrogen content of the kidney does not change.

METHODS

Rats of the Wistar strain (Carworth) were placed in individual hanging basket-type cages and allowed to eat freely a semisynthetic diet adequate for normal growth, pregnancy, and lactation (13, 14). They were weighed and fed 3 times a week. Water was available at all times. The rats were divided into groups based on similarity in dietary intake and growth rate. One rat, the experimental animal, which was to receive the tumor was allowed to eat freely. The remaining animals in each group, pair-fed controls, were fed only what the experimental rat had eaten in the preceding period. The Walker tumor was implanted aseptically by trocar into the subcutaneous tissues of the inguinal region of the experimental animal. Each pair-fed group was observed at least 2 weeks before the onset of tumor growth. The experimental animals were allowed to die or were killed at various stages of tumor growth, so that earlier effects could be noted. The pair-fed controls were killed at appropriate intervals.

* This study was aided by a grant from the American Cancer Society, on recommendation of the Committee on Growth, National Research Council.

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Received for publication, February 23, 1950.

The liver, heart, spleen, kidneys, lymphoid tissue, and the tumor were excised and freed of all fat and connective tissue. The lymphoid tissue was removed, including the thymus, the cervical lymph nodes, two groups of axillary nodes, the mediastinal nodes, the nodes draining the cecum, the lymphoid chain extending from the cecum to the root of the mesentery, pre-aortic nodes, pelvic nodes, and inguinal nodes. These lymph nodes were found in every rat, although they varied in size and number even in normal rats. The gastrointestinal tract was opened from gastric cardia to anus, cleaned, and then placed in a beaker together with the remainder of the carcass. The carcass and half of each tumor were dissolved directly in 30 per cent potassium hydroxide in 95 per cent ethyl alcohol, but other organs and tissues and the remaining half of each tumor were dried before they were dissolved. Drying was accomplished by covering each tissue with 95 per cent ethyl alcohol, heating in an oven at 105° C. for 48 hours, and drying to constant weight in a desiccator over anhydrous calcium chloride. The solutions were subsequently diluted to volume, and duplicate analyses for nitrogen and total lipids were performed on aliquots as described previously (14). Results were expressed in absolute terms and on a fat-free wet-weight basis. Among rats with tumors of comparable size there was no difference between those rats that were killed and those that died, as far as organ weights and nitrogen content were concerned. No attempt was made to control the dietary intake of the experimental animals; consequently, the varied intake levels of the different groups have caused some scattering of results.

RESULTS

The data were obtained from observations of 39 groups of rats, each group containing two or more animals that ingested identical amounts of diet. Twenty-nine of these groups contained a tumor-bearing rat to which the remaining rats in the group were paired. The remaining ten groups represented normal rats without tumors, each group containing one rat to which the others were paired.

The weight curves of the 29 tumor groups can be divided into two types: a divergent type, in which the tumor-bearing animal continued to gain weight after the onset of progressive tumor growth, despite lowered food intake, while its pair-fed control lost weight; and a parallel type, in which the weight curves of the pair-fed controls were almost parallel to those of the tumor-bearing animals. In the analyses below, no differences were noted between the groups with parallel curves and those with divergent curves.

All graphs have been plotted as a function of tumor weight expressed as a fraction of the total weight of the rat and the tumor (abscissa) and the proportionate difference between the nitrogen content of an organ in a tumor-bearing animal and the nitrogen content of the same organ in the pair-fed controls (ordinate). Thus, the pair-fed control serves as the frame of reference, in order that the influence of dietary intake may be eliminated as far as possible. The scatter exhibited by control groups of normal rats, representing the error of the method, is shown along the vertical axis.

The residual carcass, of all the tissues studied, lost the most nitrogen during tumor growth because of its comparatively large mass (Fig. 1). A decrease of only 10–15 per cent represents a considerable amount of nitrogen given up to the metabolic pool, and potentially to the tumor. The residue of the tumor-bearing rat always contained significantly less nitrogen than that of its control. This decrease in nitrogen was roughly proportional to the weight of the tumor. The differences in nitrogen content of the normal rats and their pair-fed controls were slight.

Studies of the lymph nodes revealed a constant and striking decrease in their nitrogen content in tumor-bearing animals soon after the inception of tumor growth. Differences as great as 70–90 per cent were observed in some cases (Fig. 2).

The differences observed in the nitrogen content of the heart appeared to be within the error of the method except for some animals with very large neoplasms. Thus, it seems that some nitrogen may be contributed to the metabolic pool by the heart.

No differences between the nitrogen content of the kidneys of tumor-bearing animals and that of their pair-fed controls could be detected.

The rodent spleen is notoriously subject to wide variations, even among normal animals. Control observations indicated a difference in nitrogen content of ± 40 per cent even among rats of the same age, weight, and sex which had ingested identical amounts of the same diet for periods of weeks. The spleens of tumor-bearing rats in general contained appreciably more nitrogen than did their pair-fed

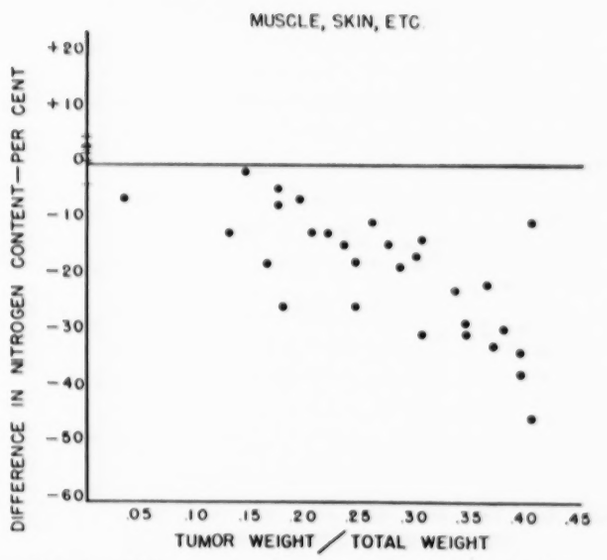

FIG. 1.—Difference in nitrogen content of "residual carcasses" of rats bearing Walker carcinoma 256 and pair-fed control rats of same age and sex at various stages of tumor growth. Dietary intake variable among pairs.

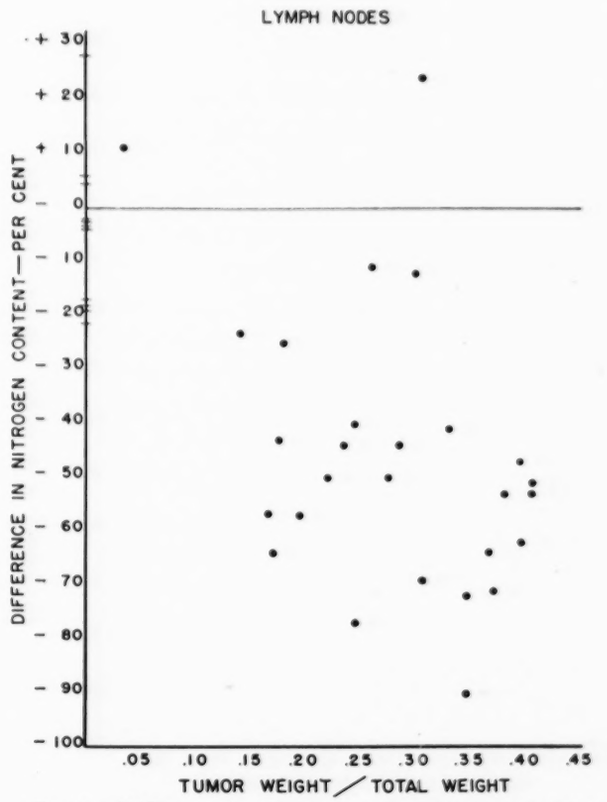

FIG. 2.—Difference in nitrogen content of lymph nodes of rats bearing Walker carcinoma 256 and pair-fed control rats of same age and sex at various stages of tumor growth. Dietary intake variable among pairs.

controls. The difference was more pronounced among rats with tumors constituting 15–30 per cent of the total weight than among rats with larger neoplasms. This suggests that a transitory period of splenomegaly, probably due to extramedullary hematopoiesis, may result from tumor growth. Some rats bearing the largest tumors had decidedly shrunken spleens. There is no direct evidence that these spleens were ever large, but this may be inferred from failure to find comparable changes among normal rats or those with Walker tumors of moderate size.

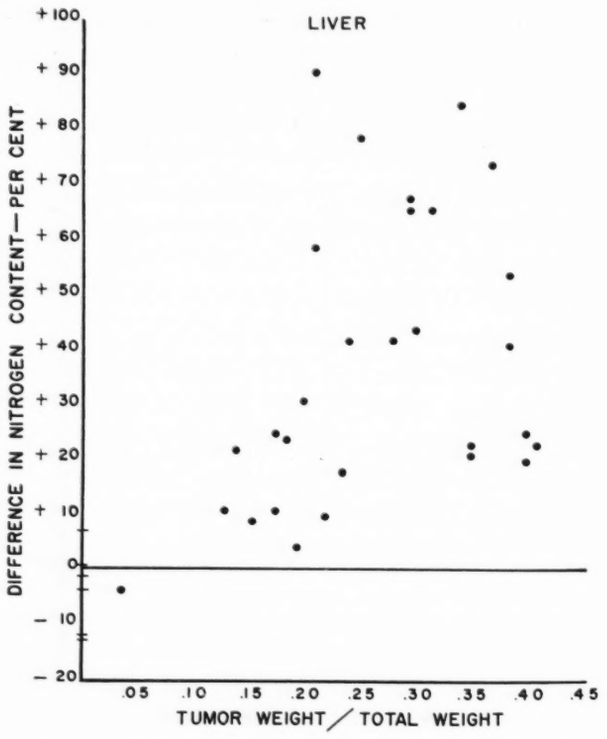

FIG. 3.—Difference in nitrogen content of lymph nodes of rats bearing Walker carcinoma 256 and pair-fed control rats of same age and sex at various stages of tumor growth. Dietary intake variable among pairs.

The livers of tumor-bearing animals usually contained significantly more nitrogen than those of their pair-fed control rats (Fig. 3). The greatest increases were in the range of the medium-sized tumors (15–30 per cent), while there seemed to be a return toward the control values when the tumors reached more than 35 per cent of total weight. That this was an actual hypertrophy is shown by the high values for liver nitrogen per gram of fat-free body weight (rat plus tumor) and even higher values for liver nitrogen per gram of carcass weight (rat minus tumor). Just as in the graph for liver nitrogen, and the graphs for liver nitrogen per gram fat-free body weight and for liver nitrogen per gram fat-free carcass weight, the nitrogen values are higher in tumor animals. Furthermore, Table 1 shows that in tumor-bearing animals liver nitrogen forms a larger proportion of total nitrogen than in the pair-fed controls and in normal rats. It is to be noted, also, that in those pair-fed animals representing partial starvation, the ratio of liver nitrogen to total nitrogen was low. This is in marked contrast to the results with tumor-bearing animals.

The scatter in Figure 3 is ascribed to differences in dietary intake of individual groups of rats, for the degree of anorexia varies. Larger groups of rats were studied in which each rat ate the same amount of diet. Only those rats whose tumors grew at approximately the same rate were used. Tumor-bearing animals and their nontumorous pair-fed controls were sacrificed at suitable intervals and the nitrogen content of the livers compared. A typical experience is depicted in Figure 4, which supports our contention that the liver may lose nitrogen during the period of precipitous weight loss preceding death.

There was close correlation between nitrogen content and both wet and dry weights of the various organs. In other words, the same differences were found on the basis of weights as on the basis of nitrogen content, and the same conclusions could have been drawn from weight data alone. The livers of tumor-bearing animals, however, contained consistently more water (74.3 per cent ± 0.3 per cent) than those of the pair-fed controls (71.5 ± 0.3 per cent). The difference, 2.8 per

TABLE 1

NITROGEN DISTRIBUTION AS PER CENT TOTAL NITROGEN

	Liver (per cent)	Kidney (per cent)	Heart (per cent)	Nodes	Spleen (per cent)	Carcass
Addis *et al.* (4), 30 rats, pooled	4.3	0.6	0.3		94.8	
37 normal rats	4.3±0.5	0.67±0.08	0.28±0.03	0.41±0.10	0.53±0.17	93.8±0.10
32 tumor animals	5.78	0.81	0.27	0.20	0.73	92.3
32 pair-fed controls	3.56	0.66	0.27	0.34	0.42	94.8
10 pair-fed controls losing more than 10% of body weight	3.29	0.66	0.26	0.34	0.42	95.1

cent, is more than 6 times its standard error and therefore statistically significant.

The Walker tumor had a strikingly constant water content of 84.4 ± 0.1 per cent. The nitrogen content of the tumor was likewise quite constant regardless of size (14).

The distribution of the nitrogen in each organ as a proportion of total nitrogen in normal rats (i.e., rats not paired to tumor animals), gave values that agree closely with those derived by Addis and others (3, 4), who used large numbers of pooled rats and organs (Table 1). As would be expected from the observations above, the nitrogen distribution in tumor animals shows that: (*a*) the heart nitrogen remained the same as both controls and normal, (*b*) the node nitrogen was lower than both, (*c*) the spleen nitrogen was higher, and (*d*) the carcass nitrogen was slightly lower. The fact that the kidney nitrogen was slightly higher than both normals and controls is not easily explainable.

DISCUSSION

These results indicate that most of the tissues that relinquish protein (2) or lose weight (19) during the course of simple caloric starvation also yield up a fraction of their nitrogen during the period of tumor growth, with the exception of the liver, spleen, and kidneys. This strengthens the over-all concept of starvation as an important mechanism by which malignant neoplasms may kill their hosts and, as has been shown previously, the degree of starvation exceeds that imposed on the animal by anorexia alone (13). The splenic changes probably do not accurately reflect the metabolic picture in respect to over-all requirements for calories or building blocks. We have no explanation for the failure of the kidneys to lose nitrogen in rats bearing the Walker carcinoma.

Those organs and tissues that relinquished nitrogen during the course of tumor growth represent only *potential* sources of building blocks for the neoplasm. One must assume that the nitrogen was contributed to the metabolic pool, hence, became available for the general needs of the body as well as the tumor. Study of the nitrogen excretion of rats bearing Walker carcinomas and their pair-fed, nontumorous controls in this laboratory has failed to reveal any marked difference in their excretion of total nitrogen, urea, or ammonia until the *ante mortem* period of rapid weight loss. Then, most tumor-bearing rats excreted significantly more nitrogen as urea than did the pair-fed controls. Most of the nitrogen lost from tissues probably was used by the tumor under such circumstances.

The hypertrophy of the liver accompanying growth of transplanted tumors in rodents is well authenticated (5, 6, 11, 12, 18, 20), but apparently only McEwen and Haven (12) have shown previously that the liver tends to grow smaller late in the course of tumor development. The question immediately arises as to the nature of the hypertrophied liver. Greenstein (8), Lan (10), and others have shown that the activity of certain enzymes in the livers of tumor-bearing animals is decidedly different from that found in normal rats. The small difference in the water content cannot account for the increased weight which is due in part, at least, to nitrogen in some form or other, as these experiments show.

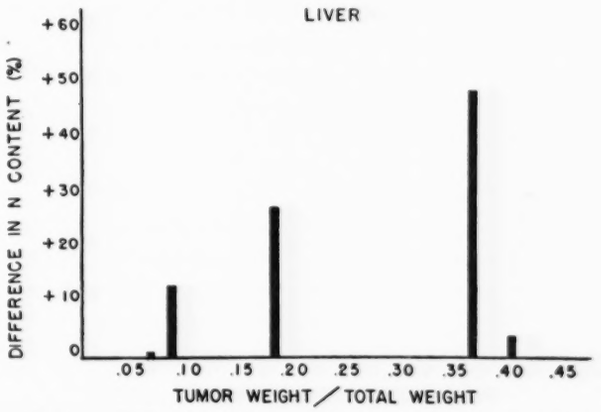

FIG. 4.—Difference in nitrogen content of livers of rats bearing Walker carcinoma 256 and pair-fed controls of same age and sex at various stages of tumor growth. Dietary intake identical in group.

A consideration of the evidence suggests that growth of the Walker tumor may be divided into three phases: (*a*) a short period during which the major part of the nitrogenous building blocks necessary for tumor formation are derived from the diet's contribution to the metabolic pool—in most cases this period ends when the tumor has attained approximately 10 per cent of the total body weight (rat plus tumor); (*b*) a period of tumor growth when the dietary intake is inadequate to furnish all of the building blocks that the neoplasm needs; dispensable stores of nitrogen in various tissues of the body are relinquished to the metabolic pool and potentially serve as precursors of tumor tissue; the onset of liver hypertrophy appears to coincide with the onset of this period; (*c*) a short *ante mortem* period of precipitous weight loss, when even the liver relinquishes the greater part of itself either to be used for tumor growth or for necessary metabolic function. During this last period most of the host's lipid stores have been depleted (13) and it may be that the nitrogenous substances lost by

the liver represent amino acids that are deaminated in order that their residues may furnish additional energy.

How far these findings apply to the generality of malignant neoplasms is conjectural. The phenomenon of liver hypertrophy in tumor-bearing rodents is adequately documented. It is well known that in certain cachectic patients dying of cancer small livers are revealed at autopsy. Abels *et al.* (1) describe liver hypertrophy as affecting 42 per cent of the patients with gastro-intestinal cancer whom they studied. Murphy and Sturm (15) found that the lymph nodes of rats bearing a transplanted lymphoma became smaller as tumor growth progressed. Homburger, on the other hand, observed an increase in the nitrogen content of the lymph nodes of mice in which either Sarcoma 180 or a transplantable mammary adenocarcinoma grew progressively (9, 17). This may represent a species difference. Less information is available with respect to the change in lymph nodes of clinical cancer. In 1906, Sampson (16) showed that the amount of lymphoid tissue in the pelves of women with uterine cancer was considerably increased, and Gilchrist and David (7) have described lymphoid hyperplasia in rectal carcinoma. Whether or not the entire lymphatic system of patients with clinical cancer is materially altered remains to be determined.

SUMMARY

The nitrogen content of the lymph nodes, livers, spleens, hearts, kidneys, and "residual carcasses" of rats bearing Walker carcinoma 256 and pair-fed noncancerous rats of the same age and sex was compared at intervals during tumor growth. The "residual carcasses" and lymph nodes of the tumor-bearing rats contained less nitrogen than did the same tissues of the pair-fed controls. The hearts lost nitrogen only among those rats with relatively huge tumors. No difference in renal nitrogen content was found between the two groups. The livers of the cancerous rats acquired nitrogen during a part of the period of tumor growth but, at death, some of them contained no more nitrogen than did the livers of the pair-fed controls.

The organs and tissues that lost nitrogen while the Walker tumor was growing represent potential sources of nitrogenous building blocks for the Walker carcinoma.

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The Virus-induced Papilloma-to-Carcinoma Sequence
I. The Growth Pattern in Natural and Experimental Infections*

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INTRODUCTION

The discovery by Shope (22) of the virus etiology of a naturally occurring epithelial tumor of wild cottontail rabbits, and the demonstration by Rous and Beard (16, 17) and others (4, 9, 24) that this benign virus-induced growth may terminate in an epidermoid carcinoma, have been followed by a wide variety of investigations of this disease and its etiological agent. Our interest in this disease and in the causative virus has been continuous since June, 1934. Since then we have kept in our rabbit colony more than 1,300 wild cottontail and domestic rabbits with papillomatosis. Our cumulative data and comparative studies, by reflecting a wide experience that has confirmed and extended work reported previously, should be of interest to those actively engaged in the field of experimental cancer research. The observations that pertain to the disease in the natural host—the cottontail rabbit—furthermore, should materially influence the theoretical implications that bear on the relationship of viruses to mammalian cancer. Our findings, therefore, will be presented, for convenience, in three communications.

Earlier published studies from several laboratories included accounts of the clinical and pathological findings of the virus-induced rabbit papilloma and of the cancer that may follow (1, 4, 5, 7, 9–11, 14–16, 22, 23). Not uncommonly, the natural sequence of changes has been interrupted by experimental procedures which were undertaken to elucidate some point at question (3, 17, 19, 20) or to accelerate the papilloma-to-carcinoma sequence (3, 17, 19, 20). However desirable such purposeful interference may be, it must modify to some extent the alterations that occur in the absence of such procedures. Early, therefore, we began to set aside a few animals from the wild cottontail and domestic rabbits with papillomatosis that were being kept in our colony, for continuous observation.

By the end of a decade, 594 rabbits had been set aside just as described. The interference by man with the natural process in each of these rabbits consisted of nothing more than the handling that was unavoidable for an animal kept under laboratory conditions and examined at weekly intervals. Among these 594 rabbits were 137 wild cottontail rabbits which had been kept for more than 6 months. We have proof for the presence of epidermoid carcinomas in 33 of these 137 cottontails. This incidence of about 25 per cent was not in keeping with the reported rarity of cancer in the natural host (2, 7, 10, 12, 13, 21). It became desirable for purposes of comparison, therefore, to include data collected concurrently from the domestic rabbits that were being kept under similar conditions. Accordingly, the present paper, the first of the series, presents our observations and data that relate to the sequence of changes and to the ultimate fate of naturally and experimentally induced papillomas in 594 rabbits.

MATERIALS AND METHODS

Rabbits.—Among 1,379 rabbits with virus-induced papillomatosis observed during the period of July, 1934, to April, 1944, were 594 rabbits that had been kept for from several months to several years without interference with the natural se-

* This investigation was aided by a grant from the Jane Coffin Child's Memorial Fund for Medical Research.

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Received for publication, February 27, 1950.

quence of alterative changes characteristic of this tumor. These rabbits fell into three groups: *Group I* consisted of 207 wild, western cottontail rabbits (*Sylvilagus floridanus alacer* Bangs) which had naturally occurring papillomas when captured by Mr. Earl Johnson of Rago, Kansas. *Group II* was made up of 94 wild cottontail rabbits with experimentally induced papillomatosis. This group included 59 rabbits, without evidence of papillomatosis when they arrived from Kansas, and 35 eastern cottontail rabbits (*Sylvilagus floridanus mearnsi* Allen) captured in northwestern New York. *Group III* was comprised of 293 domestic rabbits (genus, *Oryctolagus*), mostly of pure-bred American Dutch strain, with experimentally induced papillomatosis.

Management of rabbit colony.—Handling of the rabbits was purposely limited throughout the period of observation. Each rabbit was housed singly in a metal cage with rabbit chow, alfalfa hay, and water provided daily. The animal was removed at weekly intervals to permit cleaning or replacement of its cage and to permit examination, including careful measurement of the tumors present.

Rabbit papilloma virus.—Forty-four strains of papilloma virus were employed. The first strain was supplied as papillomatous tissue in July, 1934, by Dr. Richard Shope;[1] 22 strains were obtained from wild cottontail rabbits captured and shipped from Rago, Kansas, by Mr. Earl Johnson, and 21 strains represented laboratory-passaged virus derived from Kansas cottontails.

The tissues were triturated with alundum in Locke's solution to yield a 10 per cent suspension. Meat-extract broth was substituted for Locke's solution when filtration experiments were contemplated. (Before trituration, tissues preserved in glycerin were first washed 3 times in Locke's solution to remove excess glycerin.) This suspension was centrifuged horizontally at 1,500 r.p.m. for 30 minutes, and the supernatant fluid directly, or after filtration, was used undiluted or in decimal dilutions to inoculate animals.

Inoculation of rabbits.—Areas of skin approximately 2 cm. in diameter, on the ventral and lateral surfaces of the body, or on the inner aspect of the distal half of each ear, were prepared as sites for inoculation by removing all hair with clippers, or by shaving, followed by light cross-hatch scarification. Whenever possible, adjacent sites for inoculation were well separated by strips of hair. Inoculation was effected by rubbing 0.1 ml. of virus suspension into each scarified site, but a few rabbits were infected by endermal injection of 0.1 ml.

The extent of growth was recorded at weekly intervals for each lesion, according to a standard scale (21): 4+ = confluent papillomatosis, 3+ = semiconfluent papillomatosis, 2+ = many discrete papillomas, + = a few discrete papillomas, ± = 2 or 3 papillomas, ∓ = 1 papilloma, and 0 = negative; and according to topographic measurements in centimeters of surface area and height.

Pathological examination.—Autopsies were performed on all animals. Tissues were fixed in Zenker's (5 per cent acetic acid) fluid, sectioned, and stained by means of hematoxylin and eosin.

OBSERVATIONS

Our observations on papillomatosis in many hundreds of rabbits have yielded a composite picture sufficiently different to justify this description of the disease. It will be noted that this first paper necessarily recapitulates in part earlier descriptions, records new information, and, in general, serves as an introduction to the succeeding papers of the series.

The Growth Pattern

The growth pattern of papillomatosis, as it was observed in 594 rabbits kept without any purposeful outside interference with the natural sequence of changes, will be described first.

It was expected that a difference in the pattern of growth would be shown by the two varieties of rabbits employed. We found, however, that the common pattern of each papilloma, irrespective of host, or of host species, was predictable and fell into three phases: (*a*) the *proliferative phase*, which is characterized by multiplication at maximal rate of infected epithelial cells; (*b*) the *stationary phase*, during which multiplication of the epithelial cells in the *stratum germinativum* and their transformation to successive strata and final desquamation proceed slowly without measurable change in the gross appearance of the papilloma; (*c*) the *involutionary phase*, which is manifest for each lesion either by ultimate disappearance following progressive decrease in size, secondary to cessation of cellular multiplication and atrophy of component cells, or by radical and progressive transformation in cellular pattern to that of an anaplastic malignant tumor with secondary metastases.

[1] A sample of the papillomatous tissue received from Dr. Shope and a sample representative of each of two strains which we had recovered in 1934 from Kansas cottontail rabbits were removed in November, 1948, from 50 per cent glycerin for transfer to each of three cottontails. It was found that all three samples contained virus after 14 years of storage as proved by the appearance at all sites of inoculation of papillomas that became confluent. These results confirm the earlier findings by Fischer and Green (Proc. Soc. Exper. Biol. & Med., **64**:452–53, 1947).

(*a*) The *proliferative phase*, in our experience, became evident by the appearance of tiny vesiculoid papules following a variable incubation period of from 7 to 42 days after the virus had been brought into contact with injured epidermis. These papules resulted from active multiplication of the cells of the *stratum germinativum*. Within a few days, orderly extension took place laterally and outwardly, until after several weeks the epithelial cells, by undergoing successive changes, differentiated for most papillomas into a *stratum granulosum*, *stratum lucidum*, *stratum corneum*, and *stratum disjunctum*. Grossly, the resultant growths ranged in size from small mounds and horns a few millimeters in height to enormous verrucous masses and cutaneous horns. The lesions were discrete when they first appeared, but, if sufficient virus had been present in the inoculum, they merged within 7 to 28 days to result in a heaped-up conglomerate lesion, or a mass of partially superimposed but separable single papillomas. The most important determining factor in the proliferative phase was the size of the inoculum, for it soon became apparent that the incubation period, immediate extent of reaction, date of confluence, and ultimate size were directly related to the number of virus entities. Thus, inoculation with successive tenfold dilutions of virus elicited lesions that fell into a graded series ranging from massive confluent growths produced by the 10^1 dilution to one or two small discrete papillomas where the inoculation of a higher dilution led to the end-point. Moreover, when multiple rabbits, in response to successive dilutions of a given virus suspension each, yielded a graded series of lesions, this identity of response—by emphasizing the role of the virus—made less important the role of the host.[2] Exceptionally, and quite unpredictably, among several rabbits tested, a single rabbit reacted to infection by showing a prolongation in the incubation period, a decreased tissue proliferation, and early involutionary changes with disappearance of the lesion. When an occasional domestic rabbit or cottontail captured in New York showed such evidence of resistance to infection, an explanation was sought. No evidence as shown by the presence of natural neutralizing antibody, however, was found when samples of serum withdrawn before and after infection were tested for virus-neutralizing antibodies (25). On the other hand, the resistance commonly shown by the cottontails from Kansas was attributed to residual immunity, acquired through previous natural infection. As it was impossible to rule out previous infection in cottontails captured in enzoötic areas, we have been skeptical of an interpretation which was founded on the alterations that occur in western cottontail rabbits.

(*b*) The *stationary phase* bridged the proliferative and involutionary phases. Alterative changes were so gradual that the evolution of successive phases became apparent largely in retrospect. Desquamation of superficial layers of epithelium proceeded apace with replacement of underlying strata by a slowly proliferating *stratum germinativum*. Grossly, the lesions commonly appeared quiescent without dessicative or degenerative changes, such as usually characterize the involutionary phase.

(*c*) The *involutionary phase* terminates the growth cycle. It seems that failure of the germinal layers to multiply results in gross regression, ultimate disappearance of the lesion, and natural termination of the process. When malignancy supervened, on the other hand, vigor of growth was renewed with replacement of papilloma cells by anaplastic cells to result in gross and microscopic transformation to an epidermoid carcinoma.

Data Relating to the Incidence of Successive Structural Changes

In Tables 1, 2, and 3 the status of progressive alterative change for 594 rabbits is indicated by showing the incidence according to month of disease. The rabbits are allocated by serial letter: Series A, the cottontail rabbits that had papillomas when they arrived from Kansas; Series B, cottontails captured in New York or Kansas and infected experimentally; Series C, domestic rabbits infected experimentally.

It will be noted in Table 1 that it is indeed rare for papillomas to continue for more than 18 months as benign growths. The critical period for change was found to occur for the natural infections of cottontails and for the experimental infections of domestic rabbits at about the ninth month and for the experimental infections of cottontail rabbits at about the twelfth month. Since the naturally infected western cottontails had well developed papillomas when they came under observation, it is probable that the reactivity of all cottontails is the same and that benign lesions in this host persisted longer than in domestic rabbits.

Table 2 shows the incidence by month of the spontaneous involution of the disease. It can be

[2] It is suggested that the number of virus particles brought at the time of infection into immediate association with injured cells determines the growth pattern of any single tumor. This quantitative relationship became apparent several months after the infection of a rabbit when lesions which had been elicited by successive tenfold increments of virus ranged from actively proliferating, confluent lesions, through multiple single lesions to regression or disappearance.

seen that from 6 to 21 per cent of the lesions disappeared within the first 6 months of observation. After the sixth month, whereas more than a third of the naturally induced lesions disappeared, none of the experimentally induced lesions underwent regression. It seems questionable that this difference can be attributed to the mode of infection, seldom occurred until after the virus-induced lesions had been under observation for 6 months or longer. Accordingly, the data presented in Tables 1, 2, and 3 are recapitulated in Tables 4 and 5. Among 427 rabbits kept for less than 6 months (Table 4), the incidence of complete regression of lesions (as tabulated by rabbit series) ranged from

TABLE 1

INCIDENCE OF BENIGN PAPILLOMATOSIS BY MONTH OF DISEASE

Series	Host rabbit	Condition of infection	Period of observation in months: 0–6 No.	0–6 Per cent	6–12 No.	6–12 Per cent	12–18 No.	12–18 Per cent	18–24 No.	18–24 Per cent	Over 6 No.	Over 6 Per cent
A	Cottontail	Natural	74/95*	78	32/65	49	11/32	34	3/15	20	46/112	41
B	"	Experimental	65/69	94	16/16	100	3/7	43	0/2	0	19/25	76
C	Domestic	"	239/263	91	6/14	43	2/14	14	0/2	0	8/30	27
Total number:			378/427	89	54/95	57	16/53	30	3/19	2	73/167	44

* Denominator = Number of rabbits under observation.
Numerator = Number of rabbits with benign papillomatosis.

TABLE 2

INCIDENCE OF SPONTANEOUS INVOLUTION BY MONTH OF DISEASE

Series	Host rabbit	Condition of infection	Period of observation in months: 0–6 No.	0–6 Per cent	6–12 No.	6–12 Per cent	12–18 No.	12–18 Per cent	18–24 No.	18–24 Per cent	Over 6 No.	Over 6 Per cent
A	Cottontail	Natural	20/95*	21	24/65	37	13/32	40	3/15	20	40/112	36
B	"	Experimental	4/69	6	0/16	0	0/7	0	0/2	0	0/25	0
C	Domestic	"	24/263	9	0/14	0	0/14	0	0/2	0	0/30	0
Total number:			48/427	11	24/95	25	13/53	24	3/19	2	40/167	24

* Denominator = Number of rabbits under observation.
Numerator = Number of rabbits whose lesions had disappeared.

TABLE 3

INCIDENCE OF CARCINOMATOUS CHANGE BY MONTH OF DISEASE

Series	Host rabbit	Condition of infection	Period of observation in months: 0–6 No.	0–6 Per cent	6–12 No.	6–12 Per cent	12–18 No.	12–18 Per cent	18–24 No.	18–24 Per cent	Over 6 No.	Over 6 Per cent
A	Cottontail	Natural	1/95*	1	9/65	14	8/32	25	9/15	60	26/112	23
B	"	Experimental	0/69	0	0/16	0	4/7	57	2/2	100	6/25	24
C	Domestic	"	0/263	0	8/14	57	12/14	86	2/2	100	22/30	73
Total number:			1/427	.002	17/95	18	24/53	45	13/19	68	54/167	32

* Denominator = Number of rabbits under observation.
Numerator = Number of rabbits with malignancy.

since the incidence of involutionary regression (Table 2) is unrelated to the incidence of involutionary carcinomatous change (Table 3). Thus, as shown by the data presented in Table 3, about 25 per cent of cottontail rabbits, whether naturally or experimentally infected, developed cancers when kept for more than 6 months. The papillomatous lesions of domestic rabbits, on the other hand, showed carcinomatous alteration earlier and in greater percentage (Tables 4 and 5).

Termination of the natural growth pattern by alterative changes to regression and disappearance of lesions, or in malignancy and death of the host, 6 to 21 per cent. Cancers developed in but a single animal, a cottontail, which had well developed papillomas on arrival from Kansas. By contrast, a striking difference in the incidence of alterative change was found in the 167 rabbits with lesions older than 6 months (Table 5). The lesions underwent carcinomatous change in about one-fourth of the 137 cottontail rabbits, whether naturally or experimentally infected, and in three-fourths of the domestic rabbits. Moreover, the papillomas on more than a third of the cottontails with natural infections underwent regression and disappeared.

DISCUSSION

In the present report, the first of a series of three, the growth pattern of the epithelial tumor induced by rabbit papilloma virus (Shope) is described. The sequence of changes is well defined and predictable. Three successive phases occur: proliferative, stationary, and involutionary. The involutionary phase can terminate either in regression and disappearance, or in cancer. It is our impression that involution with disappearance of the tumor represents an abnormal aberration that occurs with sufficient regularity to establish papilloma virus as an effective carcinogenic agent. The relationship of the virus to the cancer that follows months later would appear to be analogous to the relationship of methylcholanthrene to the cancer that may follow a single application of this carcinogenic hydrocarbon. The role of the virus, therefore, seems to us to be that of a provocative carcinogenic agent and not that of an actuating carcinogen.[3]

The present findings are entirely based on the observation of animals that were kept without any interference with the natural sequence of alterative changes. This fact is important, because it has been a common practice in experimental investigations relating to the papilloma-to-carcinoma sequence to employ ancillary agents. Multiple biopsies (17, 18), tissue-stimulatory agents (Scharlach R [1, 5, 17, 18] and a turpentine-acetone mixture [20]), and carcinogenic agents (tar [6, 19, 20], benzpyrene [8] and methylcholanthrene [19, 20, 23]) have been utilized to elicit information and to condition natural changes. The experimental employment of any one of these agents introduces an alterative factor which makes hazardous any generalization, since these agents *per se* not only alter the natural sequence of changes but also give rise to trauma, inflammation, and bacterial infection, of which any or all may lead to, or may enhance, malignant change in pre-existing tumors.

SUMMARY

A study has been made of the growth pattern of naturally and experimentally induced papillomas on 594 rabbits kept for from several months to several years, without interference with the natural sequence of alterative changes. The growth cycle of the virus-induced lesions fell into three phases: proliferative, stationary, and involutionary. The termination of the natural growth pattern, which resulted in the regression and disappearance of the lesions or in malignancy followed by the death of the host, seldom occurred until after the lesions had been under observation for 6 months or longer. The employment of both domestic and cottontail rabbits showed that the rabbit host did not alter the immediate pathogenicity of the virus or of the tumor's growth pattern. A difference in host reactivity, however, was indicated by the early disappearance of the virus from domestic rabbit papillomas and by a somewhat decreased incidence of cancer in the cottontail rabbits.

TABLE 4

PAPILLOMA-TO-CARCINOMA SEQUENCE IN 427 RABBITS OBSERVED FOR LESS THAN 6 MONTHS

				Diagnosis for rabbits kept for less than 6 months					
				Benign		Complete retrogression		Carcinomatous degeneration	
Series	Host rabbit	Condition of infection	Total number	No.	Per cent	No.	Per cent	No.	Per cent
A	Cottontail	Natural	95	74	78	20	21	1	1
B	"	Experimental	69	65	94	4	6	0	0
C	Domestic	"	263	239	91	24	9	0	0

TABLE 5

PAPILLOMA-TO-CARCINOMA SEQUENCE IN 167 RABBITS OBSERVED FOR 6 MONTHS OR LONGER

				Diagnosis for rabbits kept for more than 6 months					
				Benign		Complete retrogression		Carcinomatous degeneration	
Series	Host rabbit	Condition of infection	Total number	No.	Per cent	No.	Per cent	No.	Per cent
A	Cottontail	Natural	112	46	41	40	36	26	23
B	"	Experimental	25	19	76	0	0	6	24
C	Domestic	"	30	8	27	0	0	22	73

[3] For a comprehensive review of the relationship of viruses to tumors, the reader is referred to the article, "Viruses and Tumors," by Dr. Peyton Rous in *Virus Diseases*, Cornell University Press, Ithaca, 1943.

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The Effect of Human Influenza Virus (Type A) on the Incidence of Lung Tumors in Mice*

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The etiologic role of a previous attack of influenza in human lung cancer has been under question since 1919. The pertinent evidence is histological, statistical, and clinical. The idea that this infectious disease might lead to cancer originated from the old concept that infections in general might, under certain conditions, cause cancers but more specifically from the proliferative changes that occur in the lung during recovery from influenza. Following a variable amount of necrosis and desquamation of bronchial and bronchiolar epithelium during the acute phase of the disease, a tremendous over-regeneration of these cells occurs during recovery. The hyperplasia results in intrabronchial papilloma formation, extension of epithelium into adjacent alveoli, and other features which resemble and suggest early cancer.

Study of this hyperplasia led Askanazy (1) and Winternitz *et al.* (9) to predict in 1919 and 1920, following the influenza pandemic, that an increase in the incidence of lung cancer might ensue. The latter authors state:

"In a number of cases, epithelial proliferation has been so extensive that it could not be differentiated histologically from an invasive, malignant neoplasm. There is no reason to believe that malignancy might not result from the continuous stimulation of the epithelium to proliferate, in the chronic inflammatory process of the lung in influenza, just as chronic infection in the lung of a mouse results in a much higher percentage of spontaneous neoplasms of the respiratory tract in this species than in those animals where chronic pulmonary inflammatory processes are uncommon. It will be interesting, indeed, to see whether, as a late manifestation, there is an increase in the number of now relatively rare epithelial new growths in the respiratory tract of man."

Their forecasts seemed to be borne out when lung cancer became commonly recognized during the next two decades. Many observers, however, commented that many persons with tumors had not had influenza. Nevertheless, the idea of such a relationship has persisted (3, 6). Having at our disposal a suitable means, we have experimentally studied the problem of the carcinogenicity of human influenza-A virus in mice.

The laboratory mouse is an excellent animal to use for experimentally testing the relationship of influenza to lung cancer. It is susceptible to infection by human influenza virus. During recovery from influenza the bronchial and bronchiolar epithelium undergoes cell proliferation which resembles in an exaggerated degree the changes seen in man (4). Finally, inbred strains of mice are available in which the incidence of spontaneous lung tumors is accurately known. In some strains the incidence is low, so that an increase after an experimental procedure is easily detectable. Other strains, with high spontaneous incidence, are suitable to determine whether a procedure has caused tumors to appear at an earlier age and whether the number of tumors per mouse has been increased.

EXPERIMENTAL METHODS

Mice were exposed in a chamber to a mist containing human influenza virus, type A (PR-8 strain), according to a technic previously described (4, 5). Adequacy of the exposure was recognized because of deaths from influenza from the eighth to fourteenth days and because of characteristic lesions found by sacrificing mice at selected intervals. All other mice were permitted to live out their natural life span. After death, the condition of the lungs and the number of tumors were recorded. The lungs were then fixed by intratracheal injection of Zenker-formol or formalin solution. Microscopical sections, passing transversely through both lungs and the mediastinal structures, were cut from paraffin blocks and stained with hematoxylin-eosin-azure II. Final counts of tumor were made from these microscopical sections.

The mice, equally divided as to sex, were young adults, varying from 2.5 to 4.5 months of age at the time of infection. They were of the low spontaneous lung tumor strain, C57 black, and of the high lung tumor strain A. They were obtained from the Roscoe B. Jackson Memorial Labora-

* This investigation was aided by a research grant from the American Cancer Society on recommendation of the Committee on Growth.

Received for publication, March 7, 1950.

tory; after mixing, part of each group was set aside as uninfected controls. In all, 639 mice were used, of which 351 were exposed in the chamber. One infection experiment was performed with the C57 black mice, and two separate experiments, at different levels of exposure, with the A strain. Thus, there were six experimental groups, of which three were infected and three kept as uninfected controls.

RESULTS

Influenza

Following exposure, the mice appeared normal until shortly before the first deaths occurred on the eighth day—although it was known, from animals sacrificed earlier, that almost every mouse was developing characteristic lesions. The outbreak of fatal cases was short and sharp, as illustrated in Figure 1, which demonstrates the biological homogeneity of members of these two strains to this type of challenge. The mortality, occurring between the eighth and fourteenth days, was 6 per cent in the C57 blacks and 14 per cent and 53 per cent, respectively, in two experiments with the A strain. The mortality figures also indicate adequate exposure. Many of the surviving mice appeared externally normal, but others exhibited dyspnea, which was worse on exertion, for some weeks; and some of these mice later developed an asymmetrical thorax. This was found to be consequent to atelectasis of some lobes and compensatory emphysema of others. Almost every exposed mouse sacrificed during the experiment, as well as those that died, showed the evolving lesions characteristic of influenza in this species. None of the controls showed these lesions. The subsequent mortality during the course of the experiments in both the experimental and the control mice was excessive, and it was apparently due chiefly to intercurrent liver disease.

The characteristic lesions of influenza in the mouse have previously been described (4, 8), so that the details need not be repeated. Only those lesions which might be regarded as precancerous are stressed here. The disease may be divided into three stages, consisting of an acute, short, destructive and inflammatory period, a regenerative and proliferative stage, and a permanent phase showing a variety of reparative and retrogressive conditions.

During the first week, there was damage to, or necrosis of, bronchial epithelium in the areas most severely involved, followed by slight, progressive inflammatory exudation into bronchi, alveoli, and interstitial tissues by fluid and leukocytes. These changes may be designated as bronchitis and pneumonia, both of special types (Figs. 2, 3).

During the second week these inflammatory changes continued, and regeneration of epithelium began. Epithelium rapidly proliferated into bronchi and out into adjacent alveoli. Thus, epithelial bodies, often circumscribed and tumor-like in appearance, were formed during the second week (Figs. 4, 5). These lesions were small or large, and focal, lobular, or even lobar in size and distribution. They were most conspicuous between 2–4

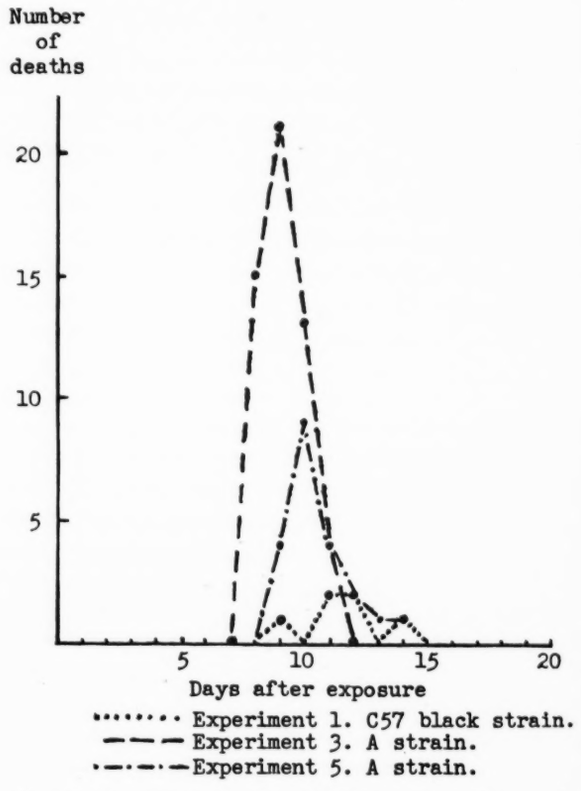
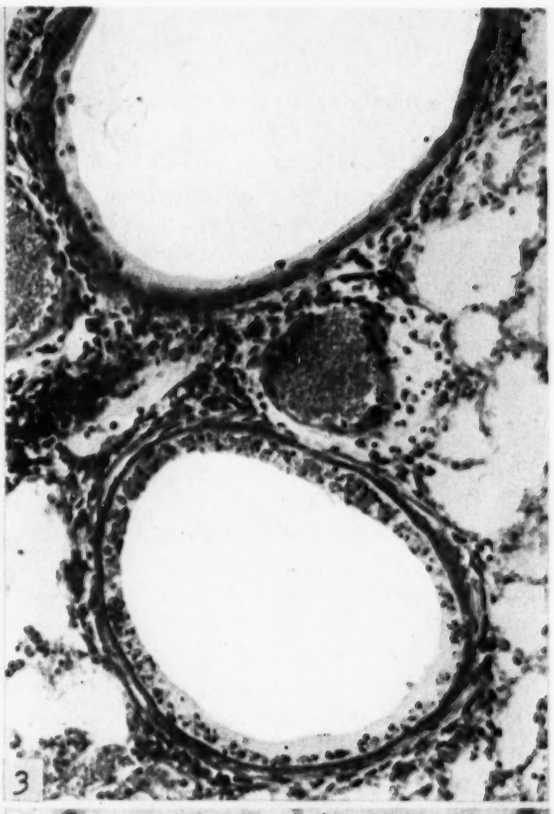
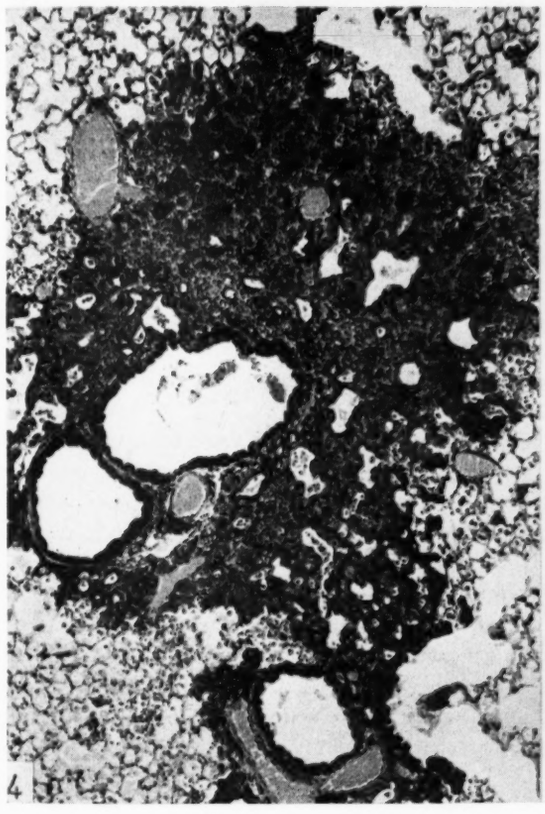


Fig. 1.—Acute influenzal deaths. Figure shows the number of deaths in sharp epidemics in three experiments. Data can be changed to per cent by using information in Table 1.

weeks. At that time they were visible to the naked eye at necropsy. They grossly resembled, to a certain degree, the common lung tumor of this species. They stood out sharply on the microscopical sections, because the acute pneumonitis elsewhere was subsiding.

After the first month reparative, retrogressive, and degenerative changes were well advanced. In mildly affected parts the inflammatory process resolved, and the bronchi and lung were restored to normal. In more severely involved areas the hyperplastic proliferated epithelium underwent gradual but great atrophy, so that by 10 months only a layer of flat lining cells was found. The most severely affected parts became atelectatic with fibrosis, bronchiectasis, and cyst formation, the latter containing mucus. Such atelectatic areas were fo-





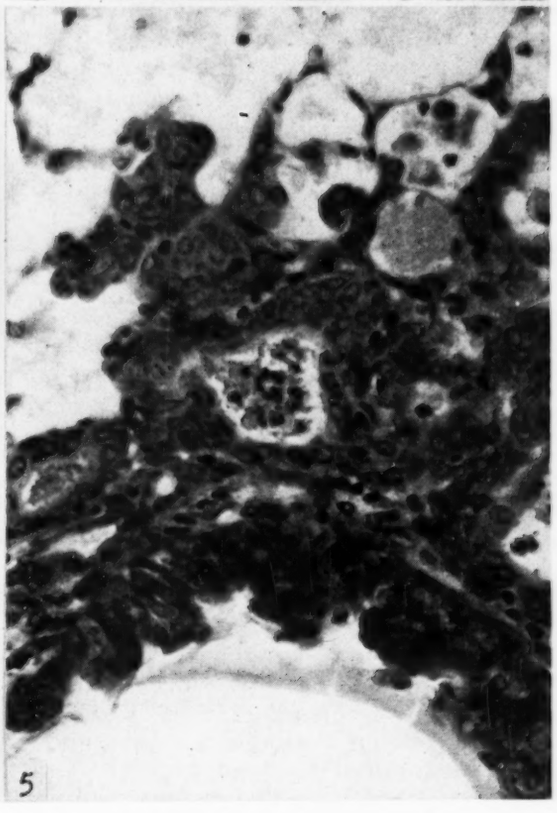


FIG. 2.—Photomicrograph (× 13) showing an acute lobular influenza 7 days after exposure. The epithelium has not yet proliferated.

FIG. 3.—Photomicrograph (× 200) showing the acute destructive bronchial lesions; 7 days after exposure. The bronchus above shows sloughing of the inner portion of the cytoplasm, leaving the nuclei; in the bronchus below the entire cells have undergone necrosis.

FIG. 4.—Photomicrograph (× 55) showing the nodular, tumor-like epithelial hyperplasia in the proliferative stage of recovery from acute influenza (14 days after exposure). Strain C57 black mouse.

FIG. 5.—High power (× 390) photomicrograph from the same mouse as shown in Figure 4 to demonstrate the papillary epithelial proliferation into a bronchus and the extension out into alveoli along alveolar walls. The cells are hypochromatic, but they appear neoplastic.

cal, lobular, or lobar, and they were shrunken and depressed (Figs. 6, 7). Microscopically, such areas showed a slight diffuse increase in fibrous tissue. The larger bronchi and some alveoli became dilated, and there was some fusion of alveoli. These nonfunctional areas occupied as much as three entire pulmonary lobes.

The sequence of events was the same in both strains of mice. In the A strain, exposed at two levels of dosage, quantitative but not qualitative differences were seen in the two groups. The chronic changes persisted throughout the life span of the mice.

Tumors

Carcinogenicity of the influenza was evaluated by comparing the following five factors in the influenza-infected and nonexposed mice: (*a*) the per cent of mice with tumor, (*b*) the average number of tumors per tumor mouse, (*c*) the number of tumors arising in influenzal versus noninfluenzal areas in the exposed mice, and (*d*) the induction time for tumors. Finally, (*e*) evidence for direct origin of tumor from influenza was sought; the histological appearance of tumors was compared with the "precancerous" influenzal-induced hyperplasias, with special emphasis on transition or intermediate forms.

C57 black strain.—No lung tumors were seen in the low lung tumor C57 black strain in either the influenza-exposed or the controls (Experiments 1 and 2, respectively). Influenza virus, therefore, did not induce lung tumors. These mice were 4 months old at the time of exposure. The survivors in the experimental group numbered 65 at 12 months of age, 30 at 18 months, and 13 at 24 months (Tables 1 and 2). The corresponding figures for the controls were 65, 18, and 5 mice. The number of long-term survivors was less than ideal, but a fairly adequate test of carcinogenicity was obtained. Tremendous epithelial hyperplasia, often nodular and tumor-like (see Fig. 4), was seen in almost every mouse examined at an early age, but this process did not eventuate in tumor. Rather, it underwent gradual atrophy. It is concluded that influenza was not a carcinogen in this strain of mice.

A strain.—In the high spontaneous lung tumor strain A mice, tumors were found in the influenza-infected and in the control groups. The per cent of mice with tumors in the two infected groups was 16.0 and 12.6 (Experiments 3 and 5 in Tables 1 and 2). The corresponding figures for the controls were 28.2 and 13.8 per cent. It is clear that the influenza did not increase the number of tumor mice, and by this criterion the virus was not a carcinogen. The number of mice with tumors was actually less in one infected group (Experiment 3) than in its control (Experiment 4). The difference is due to the greater number of tumors in the controls during the first months of the experiment. The difference is probably not statistically significant ($P = 0.2$).

The average number of tumors per tumor-bearing mouse was not greater in the infected than in the control mice. Influenza infection, although it often altered many areas in the lungs, did not increase the total number of areas from which tumors arose; and, judged by this criterion, it was not carcinogenic.

Lung tumors in mice, unlike those in man, are frequently multicentric and multiple. This affords an opportunity to study the relative incidence of tumors in influenzal and noninfluenzal areas within the same animal. While the lung tumors were studied and counted, efforts were made to determine whether they arose from normal or from influenza-altered areas. The results, given in Table 1, show that the great majority arose from normal-appearing lungs (Fig. 8). The sum of all normal areas, as seen on microscopical slides, greatly exceeded the diseased portions. An attempt was made to estimate the relative proportions of each. From a study of this data, not here presented because it was not quantitative, it was also clear that influenzal areas did not give rise to an undue proportion of tumors. One possibility, which cannot be eliminated, is that some tumors arose from small influenzal areas which became obliterated by subsequent growth of the tumor. This event is improbable, because all the small, early tumors seen were found to be originating from normal-appearing lungs (Fig. 9), and the incidence of tumors in uninfected controls was as great as in the exposed mice. By this criterion, also, influenzal infection did not induce tumors.

The age at which the first tumor appeared was less in both control strain A groups than in the infected mice (Tables 1 and 2). Judged by the induction time, influenza virus was not a carcinogen. Indeed, there is some question whether or not the virus is a tumor inhibitor or anticarcinogen. The cumulative yields and the induction times are shown graphically for the experiments with strain A mice in Fig. 10. It is clear that the virus was not carcinogenic, but uncertainty remains as to its anticarcinogenicity.

Finally, cytological study of the origins and critical comparison of the histological appearance of the epithelial hyperplasia of influenza and the lung tumors, in their early stages as well as in the fully developed lesions, showed that there were striking differences. The hyperplasia would hardly be expected to eventuate in the tumors, at least not directly as the result of failure to check the

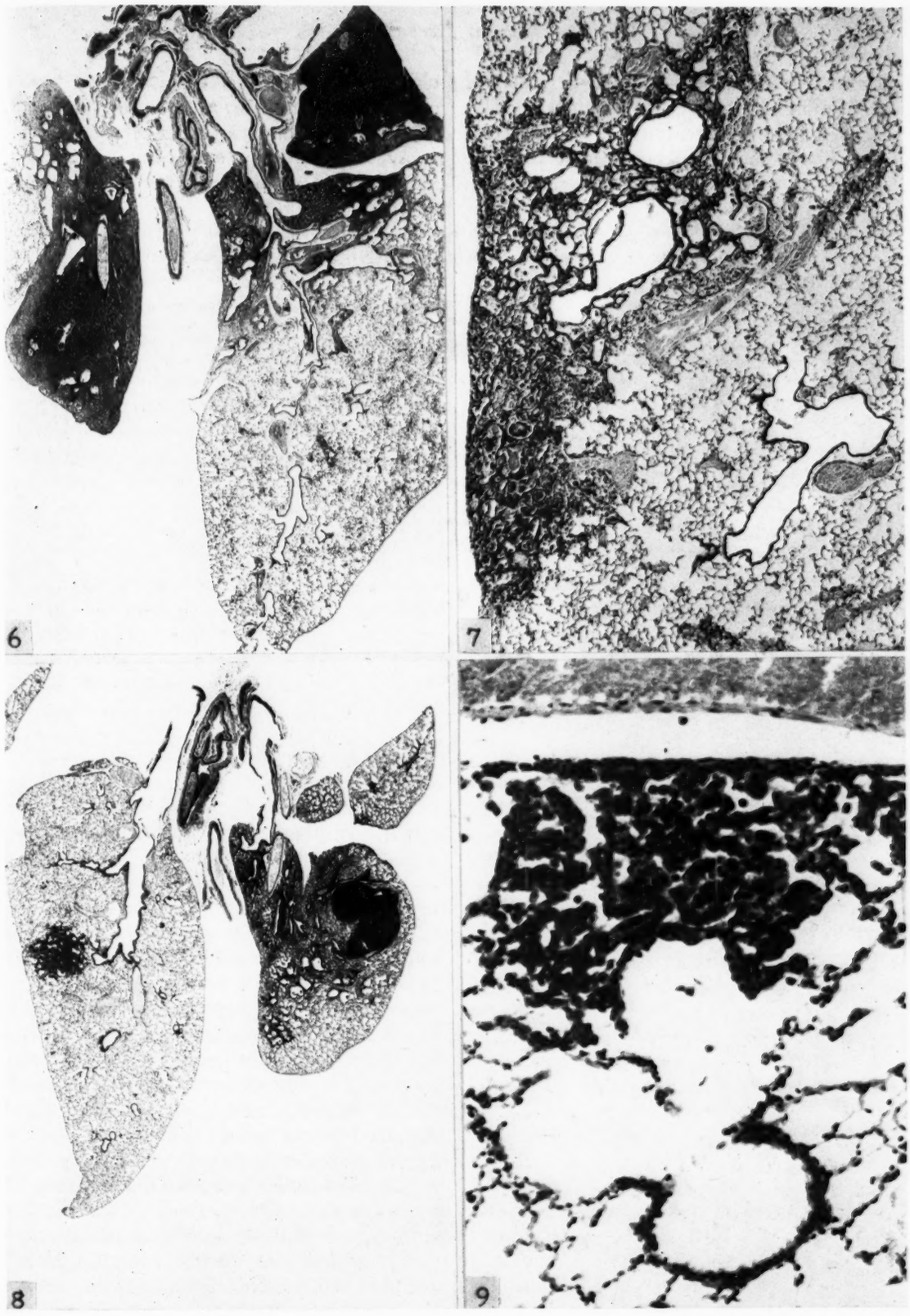


FIG. 6.—Photomicrograph (× 7) showing the lesions of chronic influenza residues at 10½ months. Strain A mouse. Three lobes are shown of which three have lesions of chronic atelectasis and two show emphysema.

FIG. 7.—Higher power (× 35) of a small chronic influenzal residue at 18 months. This is the late atrophic stage with distortion of architecture, bronchiectasis, and small alveolar cysts containing mucus.

FIG. 8.—Photomicrograph (× 7) showing two lung tumors, one originating from normal lung and the other from an influenzal lobe although not from a influenzal area.

FIG. 9.—Photomicrograph (× 200) of a very early lung tumor in an unexposed strain A mouse age 5½ months. Comparison with Figs. 4 and 5 show the differences between lung tumors and the influenzal epithelial hyperplasia described in the text.

hyperplasia. The following differences, some of which may be seen by comparing Figures 5 and 9, were observed among others: (*a*) The influenzal areas are peri-bronchiolar, lobular or lobar in shape and distribution, while the tumors are round and chiefly peripheral; (*b*) The influenza areas apparently arise from bronchi and include alveoli, whereas the tumors begin from alveolar walls away from bronchi and only later enclose the latter; (*c*) The influenzal lesions incorporate the pre-existing lung structures, while the tumors frequently displace lung tissues; (*d*) The influenzal areas show inflammation, while the early tumors do not. (*e*) The influenzal hyperplasias line alveolar walls, while the tumors are papillary, and if they occupy preexisting lung they often do so by sending papillary buds into the alveoli; (*f*) The influenzal epithelial hyperplasia is composed of fairly large, pale, hypochromatic, irregular cells which sometimes simulate a stratified squamous epithelium. It has little or no supporting stroma of its own. The tumors are composed of smaller, hyperchromatic, usually polyhedral, uniform cells arranged in single layers on a papillary stroma. Direct transition or progression of epithelial hyperplasia to tumor was not seen. Judging by these morphological criteria, the influenza was not carcinogenic, and its lesions were not precancerous.

The typical gross changes produced by influenza and the relation of subsequent lung tumors to these lesions is further illustrated by Figures 11–16. Chronic, permanent influenzal residues may exist without developing related tumors (Figs. 13 and 14). Tumors were found in normal-appearing areas of uninfected mice (Fig. 12), in the normal-appearing portions of influenza-exposed lungs (Fig. 15), or in the atelectasis of influenza residue

TABLE 1

EFFECT OF INFLUENZA ON INCIDENCE OF LUNG TUMORS IN MICE

Exp't no.	Strain of mice	Procedure	Mortality from influenza (per cent)	Age of mice when infected (months)	No. of mice at start	No. of mice effectual total	Age at first tumor (months)	No. mice with tumor	Av. no. tumors per tumor mouse	No. tumors in normal lung	No. tumors in influenzal areas	Per cent yield of tumor mice	Induction time (months)
1	C57 Bl.	Infected	6	4.0	101	83	0	0	0	0	0	0.0	0
2	C57 Bl.	None	0		83	73	0	0	0	0	0	0.0	0
3	A	Infected	53	4.5	100	38	13	6	1.3	5	3	16.0	9
4	A	None	0		103	92	5	26	1.3	36	0	28.2	1
5	A	Infected	14	2.5	150	112	10	14	1.2	16	1	12.6	8
6	A	None	0		102	87	4	12	1.1	13	0	13.8	2

TABLE 2

SUMMARY: SURVIVAL OF MICE AND OCCURRENCE OF LUNG TUMORS

Exp't no.	Strain of mice	Treatment	Survival time (in months after exposure) and mice with tumor*										Total no. of mice with tumor
			0	1†	3	6	9	12	15	18	21	24	
1	C57 Bl.	Infected	101	83	75	68	60	36	24	14	13	0	0
2	C57 Bl.	None	83	73	70	69	58	28	16	11	2	0	0
3	A	Infected	100	38	34	31	2/22	15	1/9	3/1	0		6
4	A	None	103	3/92	1/84	4/75	8/41	31	1/22	5/8	4/1	0	26
5	A	Infected	150	112	72	60	1/44	4/37	2/25	7/0			14
6	A	None	102	87	2/68	67	2/57	2/43	27	4/8	1/4	1/0	12

* The numerator is the number of mice with lung tumor in the previous months; the denominator is the number of surviving mice.

† The heavy losses were due to sacrifice and to influenza. For mortality from influenza see Table 1.

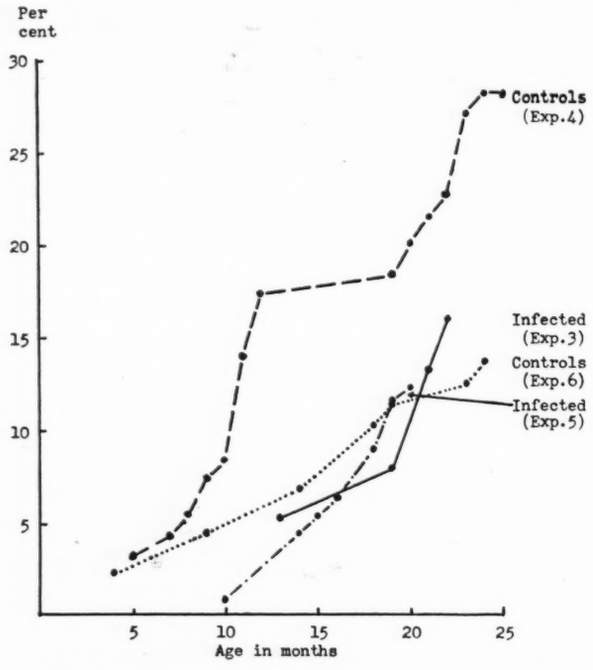


FIG. 10.—Lung tumors in influenza-infected and control mice. The points on the curves signify the number of mice that had developed lung tumors by the age indicated, expressed as a percentage of the mice surviving at one month when the acute influenza deaths had ceased. In Experiment 3 the mice were infected at 4.5 months of age; in Experiment 5 at 2.5 months.

(Fig. 16). The relative numbers of tumors under these different conditions are given in the tables and in Figure 10.

COMMENTS

In searching for agents which might induce human lung cancer, it is desirable to keep in mind not only the well known physical and chemical carcinogens which, under special conditions, cause cancers in man, but also the possible carcinogenicity of microörganisms, including viruses. It is sufficient to recall, in this connection, the role played in their respective tumors by the Shope papilloma virus, the Rous sarcoma virus, Bittner's mammary tumor agent, and the Lucké renal carcinoma virus. Studies on possible pulmonary chemical carcinogens have been reported in a previous paper (7).

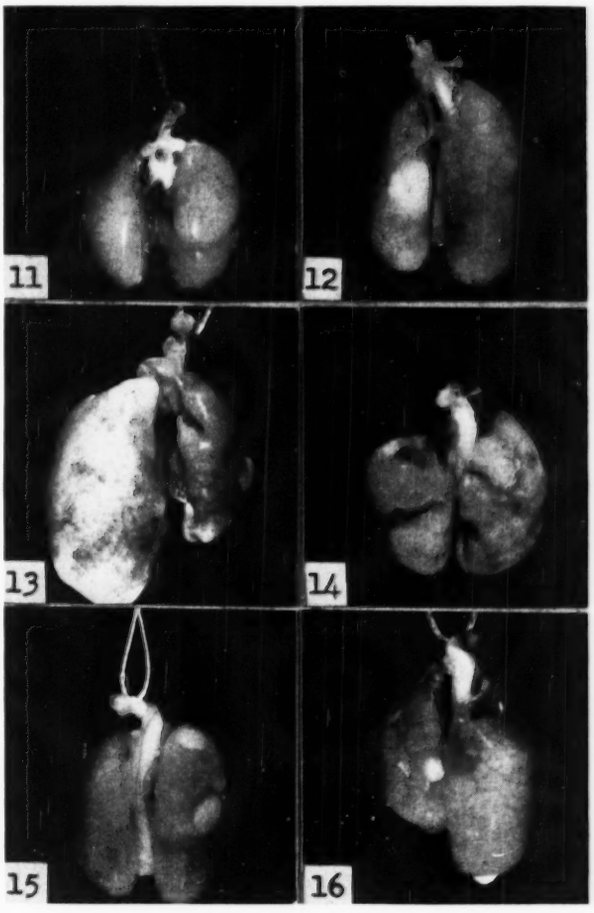
In mice, the human influenza virus was not carcinogenic according to the five criteria used for analysis. The virus was a powerful epithelial growth stimulant, however, inducing nodular hyperplasias which were suspected in mice, as they had earlier been in man, of being precancerous. Subsequent observations showed that they did not go on to tumors, and hence they were not truly precancerous. The virus was a powerful growth stimulant (i.e. an epitheliagen) but not a carcinogen. This again illustrates that cancer is not merely a simple problem in growth.

The experimental results should not, of course, be transferred to the problem in man. From all the evidence, however, there is little reason to suspect any longer that influenza does play a role in causing human lung cancer. The epithelial hyperplasias induced in the mice were relatively much greater than those described in man. One strain of mice used has a strong predisposition to spontaneous and induced lung tumors. Despite these facts, the lesions did not progress to neoplasms.

The possible inhibitory or anticarcinogenic effect of influenza virus to lung tumors deserves further study. Campbell (2) obtained evidence for the same phenomenon by a different method. He vaccinated mice by intraperitoneal injection of virus vaccine, and followed this by instillation of live virus intranasally. The mice were then exposed daily to a dust containing coal tar. He observed two tumors in mice so treated. This figure was greater than that for his controls but less than that for the dusted mice without influenza virus. The statistical significance of his results, however, is questionable.

SUMMARY

The carcinogenicity of human influenza virus (type A, PR-8 strain) for lung tumors was studied in mice. The infection produced great, tumor-like, epithelial hyperplasia during the recovery stage. Some influenza-induced lung lesions persisted throughout the life of the animals. These changes did not eventuate in lung tumor in mice of the low lung tumor strain C57 black. Neither was the infection carcinogenic nor the epithelial hyperplasia precancerous in the high spontaneous lung tumor strain A as judged by (*a*) no increase in the number of mice with tumor, (*b*) no increase in the average number of tumors per tumor mouse, (*c*) no



FIGS. 11–16.—Photographs (× 2) showing the different relations of lung tumors to the presence of chronic influenzal lesions.

FIG. 11.—Normal lung.

FIG. 12.—Normal (unexposed) lung containing a lung tumor.

FIG. 13.—Chronic lobar influenzal atelectasis on one side and compensatory emphysema on the other. Strain A mouse 7 months after infection.

FIG. 14.—Chronic focal bilateral influenzal atelectasis, without tumor. C57 black mouse 16 months after infection.

FIG. 15.—Influenza-exposed mouse without residues but with two lung tumors in normal-appearing areas. Strain A mouse 18 months after infection.

FIG. 16.—Influenza-exposed mouse with tumor arising in a lobe which shows chronic atelectasis. Strain A mouse 8½ months after infection.

greater number of tumors arising from influenzal lesions than from noninfluenzal areas, (*d*) no reduction in the tumor induction time, and (*e*) no visible microscopic transformation of influenza-induced hyperplasia to tumor.

There was a suggestion that the influenza infection might have been anticarcinogenic as indicated by a lower per cent yield of tumors (in one experiment), and a lengthening of the tumor induction time (in two experiments) in the virus-infected as compared with the unexposed control mice.

CONCLUSIONS

Human influenza virus (type A, PR-8 strain) was a powerful growth stimulant to mouse regenerating bronchial epithelial cells, but it did not induce tumors. It was a growth stimulant—an epitheliagen—but not a carcinogen. The experimental work with mice did not support the theory of a causal relationship between influenza infection and lung cancer.

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Measurements of Ketoacids in Normal and Neoplastic Rat Tissue*

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A number of ketoacids have been assuming considerable importance as intermediates in studies of carbohydrate metabolism, and as links in the interconversion of carbohydrates, fats, and proteins. It appeared that analyses for certain of these key intermediates in tissues, by *in situ* freezing under a variety of metabolic conditions, would provide very helpful information.

Several reports have appeared concerning the use of chromatography of the 2,4-dinitrophenylhydrazones as a means of separating and identifying ketoacids (1, 8, 9). The purpose of this paper is to present a method for the chromatographic separation of 2,4-dinitrophenylhydrazones of ketoacids obtained from extracts of rat tissues. By using columns of a diatomaceous material, one may vary the quantities of material over a considerable range. Very small amounts of one acid can be separated from relatively large quantities of others.

Analyses were made of certain ketoacids in a number of rat tissues. The strict precautions of the *in situ* freezing technic were used to prevent autolytic changes.

METHODS AND MATERIALS

Preliminary studies of the chromatographic method were conducted with pure ketoacid samples. Pyruvic acid was prepared from a commercial sample (Paragon Testing Laboratories) by distillation under reduced pressure, 47° C. at 4 mm. Hg, and immediate dilution of the acid to 1 M. This solution was stable indefinitely at 0° C. Oxalacetic and α-ketoglutaric acids were synthesized in this laboratory by Drs. Charles Heidelberger and W. C. Schneider. The agent used in the chromatograms was a diatomaceous earth known as Hyflo Supercel, sold by the Johns-Manville Company.

U.S.P. ether, distilled and equilibrated with water, was used in this study. It was found to be essentially free from peroxides when stored for several weeks. Dilute ether extracts of the hydrazones, with acid and with excess reagent present, were stable for 48 hours at 0° C.

EXPERIMENTAL

Chromatography.—A variety of sizes of columns from 6 to 14 mm. (inside diameter) have been used with success, the size of the column being governed by the quantity of material to be handled. The method has been applied to a problem where relatively large amounts of material were to be isolated (7).

The method was standardized, as follows: A tube of 9-mm. inside diameter with a constriction near the bottom is blocked with a small plug of glass wool and the Hyflo Supercel packed in tightly with a plunger, approximately 10 mm. at a time, until a 75-mm. column is achieved. Then a circle of paper slightly larger than the internal diameter of the tube is pushed down on top. Water-saturated ether is pipetted into the tube and light pressure from an air line applied at the top to accelerate the flow of solvent to approximately 3 ml. per minute. Approximately 15 ml. of fluid are run through the column. This packs the column evenly, removing the striations and air bubbles. It is desirable to run the chromatography in a room at 24° C. or below to avoid "channeling" due to vaporization of the ether. The column is never allowed to go dry. The solution of hydrazones in 10–16 ml. of ether (see preparation of sample) is put on the column, after easing off the pressure gradually by use of a T-tube and clamp in the air-pressure line. When the last of this hydrazone solution is just entering the column, approximately 6 ml. of solution No. 1[1] are added. When this has entered the column, 6 ml. of

* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

Received for publication, March 10, 1950.

[1] Solution No. 1: 100 ml. water-saturated diethyl ether + 1.0 ml. of 95 per cent ethanol equilibrated with 2.5 ml. of 2.0 N HCl.

Solution No. 2: 100 ml. of water-saturated diethyl ether + 2.0 ml. of 95 per cent ethanol equilibrated with 2.5 ml. of 2.0 N HCl.

Solution No. 3: 100 ml. of water-saturated diethyl ether + 3.0 ml. of 95 per cent ethanol equilibrated with 2.5 ml. of 2.0 N HCl.

solution No. 2 are added. Then the chromatography is continued, with solution No. 3 as solvent, using a flow rate of 2–3 ml/min. The excess 2,4-dinitrophenylhydrazine goes through the column with the solvent. In a typical experiment with a tissue extract containing 20 μg. of α-ketoglutaric acid, 2 μg. of oxalacetic acid, and 15 μg. of pyruvic acid, the α-ketoglutaric acid hydrazone resides in an intense 5-mm. band about 10 mm. from the top of the column, after the excess reagent is washed out; the oxalacetic acid hydrazone is a 1-mm. band

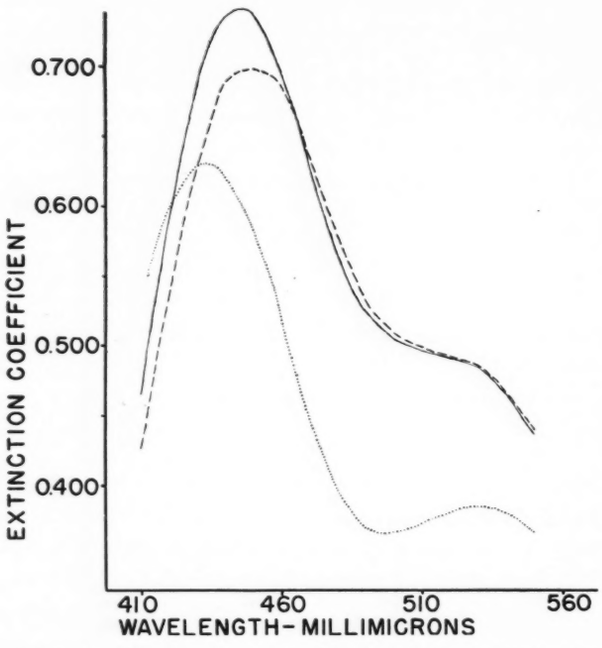

FIG. 1.—Absorption curves of 2,4-dinitrophenylhydrazones of ketoacids measured in 1.0-cm. cells with a Beckman DU quartz spectrophotometer: ———, pyruvic; ......, oxalacetic; . . . . . , α-ketoglutaric.

10–12 mm. below the first band; and the pyruvic acid hydrazone is a slightly more diffuse 10-mm. band some 10–15 mm. below the oxalacetic hydrazone. As elution is continued, the pyruvic hydrazone draws away from the other two and moves approximately twice as rapidly as that of α-ketoglutaric acid. The ether solution of each hydrazone is collected separately in a graduated (15-ml.) tube. These are each treated, in turn, with 2.8 ml. of 1.25 N NaOH and shaken. This gives an aqueous layer of 3.0 ml. If the solution is too concentrated, it is diluted with 1.25 N NaOH. The alkaline solution is drawn off into a cuvette and the absorption curve examined in a Beckman model DU quartz spectrophotometer. Within the 12–15 minutes required to accomplish this, no instability is evident. The hydrazone solution can be acidified and extracted into ether again, if desired.

The ether must be distilled to remove the variable content of alcohol and acidity. Both alcohol and acid tend to "titrate" the capacity of the columns. It is necessary to have a slight acidity in the ether to give the column good resolving ability. The addition of alcohol to the eluting solvent keeps the hydrazone bands well defined and moves the bands off more rapidly. The column described above can generally be run in 30 minutes. It is not possible to use a 3 per cent addition of alcohol without the intermediate concentrations, since this tends to run the bands together.

The bands are most easily seen and followed in indirect daylight. Under the best of conditions, bands due to as little as 0.8 μg. of ketoacid can be followed on the standard column described; up to 40 μg. of each acid can be resolved on such a column.

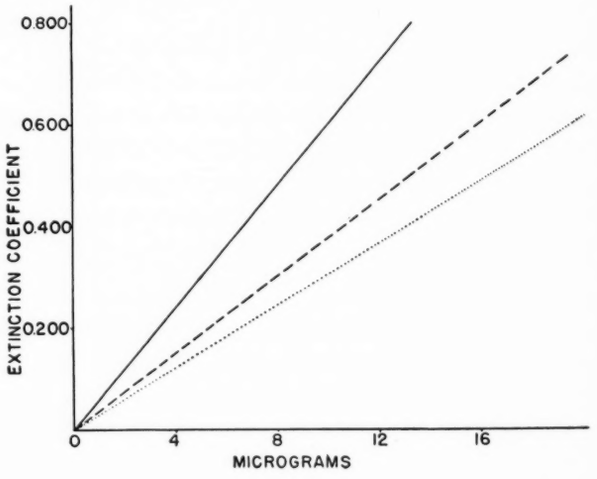

FIG. 2.—Standard curves for 2,4-dinitrophenylhydrazones of ketoacids plotted in micrograms of ketoacid used against extinction coefficient measured at the absorption maximum in a 1.0-cm. cuvette in a Beckman DU spectrophotometer: ———, pyruvic measured at 445 mμ; ....., oxalacetic measured at 450 mμ; . . . . . , α-ketoglutaric measured at 432 mμ.

Preparation of sample.—The precautions necessary to prevent autolytic changes in tissue metabolites have been discussed previously (4–6). Holtzman albino rats of both sexes, weighing 150–290 gm. were used in this study. Except where otherwise specified, they were anesthetized with nembutal (50 mg/kg body wt. intraperitoneally) and frozen in liquid air. The desired tissues were dissected out in the frozen state, ground to a powder, and inactivated in 4 per cent perchloric acid. Sufficient perchloric acid solution to make 3.0 ml. per gram of tissue was added, and the sample was broken up with a loose homogenizer pestle to mix and aid in extraction. The tissue residue was centrifuged, and the clear supernatant fluid taken. In the standardized procedure 8.0 ml.

of extract, representing 2.0 gm. of tissue, are mixed with 2.4 ml. of a solution containing 1.0 mg. of 2,4-dinitrophenylhydrazine per milliliter in 2.0 N HCl, and incubated 20 minutes at 25° C. This converts the pyruvic, oxalacetic, and α-ketoglutaric acids to the corresponding hydrazones (2). It was necessary to pool tissues from several animals, in some cases, to obtain this amount of material. The solution of hydrazones is extracted with approximately twice its volume of water-saturated ether, and the ether layer is washed with 0.5 vol. of distilled water. The ether solution is used for the chromatographic separations.

Hydrazones from pure samples of pyruvic, oxalacetic, and α-ketoglutaric acids were chromatographed and absorption curves determined. These are shown in Figure 1. The chief absorption maxima for the range studied were 445, 450, and 432 mμ for the hydrazones of pyruvic, oxalacetic, and α-ketoglutaric acids, respectively. Known amounts of each acid were treated in the same manner as were the samples of tissue extract to be assayed, and standard curves were constructed using the wave length of the absorption maximum in each case. These standard curves are illustrated in Figure 2. Approximately 97 per cent of the hydrazone was recovered in each case, the loss representing hydrazone left in the water layer on the first ether extraction. Recoveries from the columns were complete. Under circumstances where resolution was very good, the pyruvic acid hydrazone separated slightly into two bands with identical absorption spectra. This separation may have resulted from resolution of trans- and cis-isomers.

RESULTS

In most tissue samples examined by this method, all three of these ketoacids were found. The level of oxalacetic acid was below detection in brain and in blood, even when larger amounts of tissue were used. No effort was made to determine acetoacetic acid, although it gives a hydrazone band which moves off the columns ahead of that of pyruvic acid. Chromatographing tissue extracts treated as described results in several other discernible bands. Further study will be needed to identify these.

Identification of bands was accomplished routinely by use of the position of the band on the column and the absorption maximum of the alkaline solution. In at least one case for each tissue, an additional confirmation was obtained by mixing each separate band eluted from the column with the known ketoacid hydrazone. This mixture was then run on a new column to determine whether one or more than one band was obtained. When the "mixed chromatograph" gave only one band, this was interpreted as evidence of identity with the known compound.

Results of experiments in which rats were frozen in liquid air, with and without nembutal anesthesia, are presented in Table 1. These data indicated that the brief period of stimulation before the tissues were frozen (5–20 seconds) led to considerable increases among unanesthetized animals, especially in pyruvic acid. The subsequent experiments were, therefore, conducted with anesthetized rats.

Since it is convenient, when a number of tissues are obtained in an experiment, to store some of them for periods of days or weeks, a study was made of the ketoacid levels in tissues stored at

TABLE 1

THE EFFECTS OF NEMBUTAL ANESTHESIA ON KETOACID LEVELS IN TISSUES OF FED RATS FROZEN IN LIQUID AIR

	Tissue	No. of samples	Pyruvic acid (mg., per cent)	Oxalacetic acid (mg., per cent)	α-ketoglutaric acid (mg., per cent)
With anesthesia	Liver	4	0.17	0.057	0.16
	Flexner-Jobling rat carcinoma	3	1.65	0.059	1.35
No anesthesia	Liver	4	1.21	0.139	0.30
	Flexner-Jobling rat carcinoma	3	1.85	0.062	1.95

−22° C. in a freezing compartment. Pooled samples of tissue were ground in the frozen state and divided into aliquots. One aliquot was analyzed immediately. Others were stored and removed at intervals for analysis. The results of two such experiments are given in Table 2. From these data it appears that low temperatures do not entirely eliminate the reactions involving ketoacids, as they do the hydrolytic reactions involved in phosphorylating glycolysis (5) and that results are probably not reliable on samples stored more than 4 days.

The measurement of these three ketoacids may give interesting information concerning metabolic changes. Table 3 presents the results of three experiments in which fed rats were compared with 24-hour fasted rats. Pyruvic and α-ketoglutaric acids in the livers of fasted rats were markedly reduced. This may relate to a change in the balance of carbohydrate and fat metabolism.

In Table 4 data are presented on the analyses for ketoacids in a number of rat tissues, including neoplastic tissues and blood. For the examination

of blood, rats were anesthetized with nembutal, opened by an abdominal incision, and blood was taken from either the aorta (arterial) or from the inferior vena cava (venous) approximately opposite the kidneys. The blood was immediately ejected into perchloric acid and extracted as were the other tissues. It is notable that the venous blood had an elevated pyruvic acid level but that the α-ketoglutaric acid level remained the same as that in arterial blood. Oxalacetic acid, if present, was below the level of detection. The level of pyruvic acid found in arterial blood agrees well with that found by Friedemann and Haugen (2) in more extensive analyses of blood.

Two bands which appeared below the pyruvic acid hydrazone in the chromatograms obtained from tissue extracts had absorption maxima at approximately 435 mμ. In this respect, the adsorbed compounds resembled the α-ketoglutaric acid hydrazone. They were present in sufficient amount to emphasize the need for chromatographic separation before analysis.

The levels of α-ketoglutaric and pyruvic acids reported for liver are in some doubt, since they were low, relative to the levels in blood, and this organ tends to be engorged with an appreciable level of blood. The high level of α-ketoglutaric acid in kidney and in the tumors may have significance relative to amino acid synthesis, since the amination of this ketoacid has been indicated to be an entree for amino acid formation (3). The high level of pyruvic acid in the tumors reflects the high levels of lactic acid accumulated there (5).

TABLE 2

EFFECTS OF STORAGE AT −22° C. ON KETOACID LEVELS IN RAT TISSUES

Two experiments were carried out with pooled samples. Each figure is the average of two analyses on separate pooled samples.

Time of storage (days)	Tissue	Pyruvic acid (mg., per cent)	Oxalacetic acid (mg., per cent)	α-ketoglutaric acid (mg., per cent)
0	Liver	0.15	0.115	0.25
	Kidney	0.32	0.045	2.05
	Flexner-Jobling carcinoma	1.45	0.102	1.85
4	Liver	0.16	0.077	0.21
	Kidney	0.61	0.125	2.55
	Flexner-Jobling carcinoma	1.50	0.085	1.87
14	Liver	0.12	0.050	0.15
	Kidney	1.60	0.120	2.20
	Flexner-Jobling carcinoma	1.25	0.080	1.73
75	Liver	0	0	0
	Kidney	0	0	0
	Flexner-Jobling carcinoma	0.65	0.075	0.31

TABLE 3

COMPARISON OF KETOACID LEVELS IN THE LIVERS OF FASTED AND FED RATS

Each figure is the average of analyses on samples from three animals.

Ketoacid	Fed (mg., per cent)	Fasted (mg., per cent)
Pyruvic	0.30 (0.23–0.40)	0.11 (0.08–0.14)
Oxalacetic	0.11 (0.08–0.12)	0.080 (0.060–0.100)
α-ketoglutaric	0.42 (0.29–0.56)	0.17 (0.13–0.20)

SUMMARY

1. A method is described for chromatography of ketoacids in the form of their 2,4-dinitrophenylhydrazones.

2. The method is used to determine pyruvic, oxalacetic, and α-ketoglutaric acids in extracts of rat tissues which were frozen *in situ* with liquid air.

3. Analyses of tissues from animals frozen with and without nembutal anesthesia indicated the necessity of anesthesia to prevent changes due to

TABLE 4

ANALYSES FOR KETOACIDS IN THE TISSUES OF FED RATS

Tissue	No. of samples	Pyruvic acid (mg., per cent)	Oxalacetic acid (mg., per cent)	α-ketoglutaric acid (mg., per cent)
Brain	3	0.77 (0.74–0.80)	<0.02	1.25 (1.23–1.32)
Heart	3	0.29 (0.24–0.31)	0.102 (0.078–0.110)	0.21 (0.20–0.23)
Kidney	8	0.29 (0.24–0.35)	0.079 (0.05–0.100)	2.35 (1.78–3.20)
Liver	14	0.14 (0.10–0.20)	0.11 (0.080–0.122)	0.26 (0.20–0.45)
Muscle	3	0.71 (0.69–0.73)	0.093 (0.076–0.103)	0.28 (0.27–0.29)
Flexner-Jobling carcinoma	12	1.50 (1.00–1.80)	0.085 (0.05–0.100)	1.75 (1.20–2.32)
Jensen sarcoma	3	1.55 (1.30–1.65)	0.035 (0–0.100)	0.78 (0.75–0.85)
Walker 256 carcinosarcoma	4	1.95 (1.75–2.15)	0.028 (0–0.085)	1.15 (1.05–1.22)
Arterial blood	2	1.19 (1.15–1.23)	<0.02	0.50 (0.49–0.51)
Venous blood	2	3.20 (3.10–3.30)	<0.02	0.49 (0.48–0.50)

stimulation of the tissues on contact with the liquid air.

4. Fasting was found to decrease these ketoacids in rat liver.

5. Pyruvic acid was found to be very low in normal tissues and somewhat elevated in venous blood and in neoplastic tissues.

6. Oxalacetic acid was very low in all tissues studied and was not detectable in blood or brain samples.

7. α-Ketoglutaric acid was highest in kidney and in neoplastic tissues. The levels in most other tissues did not exceed that in blood.

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Book Reviews

Proceedings of the First National Cancer Conference. Sponsored by the American Cancer Society and the National Cancer Institute, 1949. Pp. 310.

This book contains the proceedings of the cancer conference which was held February 25–27, 1949, at Memphis, Tennessee. The first third of the book is devoted to publication of the nine formal talks which were presented by Drs. Sewall Wright, G. D. Snell, J. J. Biesele, A. F. Blakeslee, Paul Weiss, M. R. Irwin, C. R. Moore, F. L. Hisaw, and Alexander Haddow under the general heading "Biological Phenomena of Growth." These are followed by the addresses by Drs. K. C. D. Hickman and Bernard Cohen. The records of panel discussions on tumors of the reproductive tracts and breast, lymphoblastomas, tumors located on the head and neck, lung cancer, soft-part tumors, bone tumors, tumors of the nervous system, epidemiology, professional education technics, cancer services and facilities, cancer registration, and administration of cancer grants comprise the major share of the book. As a whole, the value of the volume is greatly diminished by the relative lack of references to original literature. Of the first nine talks, three contained no literature citations. Most of the recorders of the panel discussions made a laudable effort to credit remarks to the individual commentators, but some of the panel discussions (notably that on tumors of the breast and reproductive tract) are quite deficient in this respect. The volume may serve as a useful record for those who attended the conference, but will have less value for other members of the profession.

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Advances in Surgery, Volume 2. William DeWitt Andrus, Chairman, Editorial Board. 1st ed. New York: Interscience Publishers, Inc., 1949. Pp. 590. $11.00.

This is the second of a series of articles on surgical subjects where interest or advance has been particularly prominent in the past few years. The Editorial Board consists of ten internationally famous surgeons under the chairmanship of Dr. William DeWitt Andrus. There are eleven contributions by fourteen authors and coauthors. The chapter titles are: pulmonary function studies in relation to chest surgery; advances in surgery of the esophagus; surgical treatment of hypertension; portal bed block and portal hypertension; surgery of congenital anomalies of the heart and great vessels; recent advances in the treatment of craniocerebral injuries; use of streptomycin in the treatment of surgical infections; advances in skin grafting; the pathology of tumors of the peripheral nerves; testis tumors; and advances in the management of pancreaticoduodenal cancers.

The articles on tumors of the peripheral nerves and tumors of the testis are, as the preface states, authoritative monographs on these subjects. Most of the other articles contain excellent reviews of the earlier work in that particular field and discuss the advances up to about 1948. By the use of addenda and last-minute revision, some of the authors were able to bring their review of the literature up to early 1949. Although any book on recent advances is necessarily months behind the current literature and clinical experience, it is unfortunate that the article on congenital anomalies of the heart and great vessels has almost nothing on coarctation of the aorta.

The chapter on surgery of the esophagus by Dr. R. H. Sweet is excellent, as is Dr. C. G. Child's article on pancreaticoduodenal cancers. The discussion of the treatment of craniocerebral injuries is detailed, including the physiopathology, diagnosis, and methods of treatment as modified by the experiences gained in World War II.

Although the work of many authors, the chapters are uniformly well written and profusely illustrated, with adequate and, in some cases, exhaustive bibliographies. This book will be valuable for postgraduate surgeons and also useful to medical students, interns, and residents in bringing them a careful evaluation and discussion of the recent advances in these important subjects. For all readers it will give a good perspective and understanding of the history and more recent developments in the field that cannot be obtained in the usual surgical textbook. The reviewer heartily recommends this book.

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